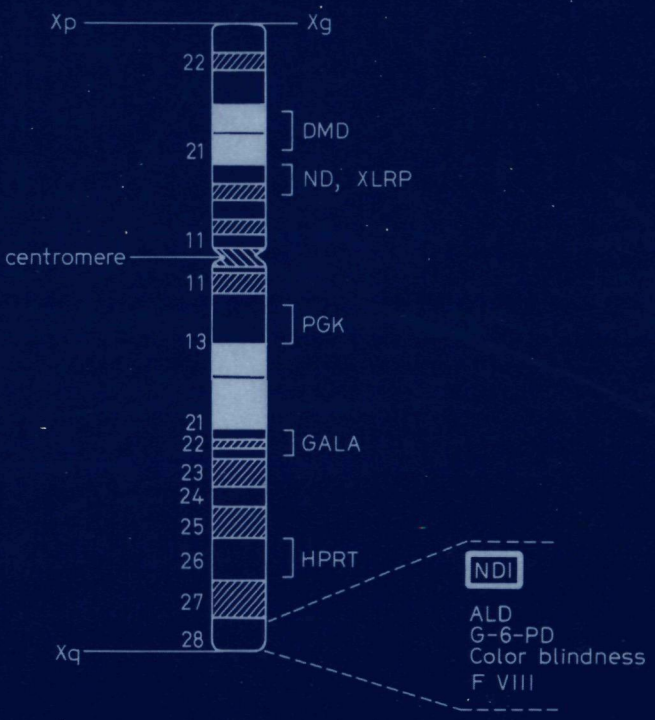
Nephrogenic Diabetes Insipidu



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Nephrogenic Diabetes Insipidus

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Cover: Map of the X chromosome showing the position of the gene locus for Nephrogenic Diabetes Insipidus.

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CHAPTER 1

GENERAL INTRODUCTION - NEPHROGENIC DIABETES INSIPIDUS : THE CLINICAL PICTURE - PATHOPHYSIOLOGY

1.1 CONGENITAL NEPHROGENIC DIABETES INSIPIDUS: THE CLINICAL PICTURE

1.1.1 HISTORY

The renal type of diabetes insipidus was appreciated as a separate clinical entity more then 40 years ago, when it was described independently by two investigators: Forssmann (1945) in Sweden and Waring et al. (1945) in the United States. In the next 15 years about 120 additional examples of the disorder were recognized and studied (Kaplan et al., 1959). Families with this type of defect, however, had been described many years before (Mc Ilraith, 1892), without being distinguished from the central or neurohormonal diabetes insipidus. The name 'Nephrogenic Diabetes Insipidus' was coined by Williams and Henry (1947), who noticed that injection of the antidiuretic hormone in doses, sufficient to induce systemic side effects, could not correct the concentrating defect. Subsequent studies revealed biologically active hormone to be present in the serum (Holliday et al., 1963) and urine (Luder and Burnett, 1954) of affected persons and lend further support to the theory of renal vasopressin unresponsiveness.
Nowadays the name 'Nephrogenic Diabetes Insipidus' is used synonymously with the terms 'vasopressin or ADH-resistant diabetes insipidus 'or 'diabetes insipidus renalis'.

1.1.2 DEFINITION AND CLINICAL MANIFESTATION

Congenital Nephrogenic Diabetes Insipidus (NDI) is a rare inherited kidney disorder, characterized by insensitivity of the distal renal nephron to the antidiuretic effect of vasopressin (Williams and Henry, 1947). As a consequence, the kidney loses its concentrating ability and produces large volumes of hypotonic urine (50-100 mosm/kg H_2O), which may lead to severe dehydration and electrolyte imbalance (hypernatremia and hyperchloremia). The osmolar urine/plasma (U/P) ratio remains constantly below 1 and cannot be reversed by exogenous application of vasopressin. As mentioned in the initial

descriptions by Waring et al. (1945), Forssman (1945), and Williams and Henry (1947), the defect in congenital NDI is present from birth and therefore manifestations of the disorder emerge within the first weeks of life. Polyuria, as high as 500 ml per day or even higher in infants, and excessive thirst are the most typical symptoms but they may not be recognized immediately (Gautier and Prader, 1956; Kaplan, 1987). Instead, the clinical picture is dominated by signs of chronic dehydration. Irritability, poor feeding and poor weight gain are often the initial symptoms. Patients are eager to suck, but may vomit during or shortly after the feeding. Dehydration is evidenced by dryness of the skin, loss of normal skin turgor, recession of the eyeballs, increased periorbital fold depth, recession of the anterior fontanelle and scaphoid abdomen. Intermittent high fever is a common complication of the dehydrated state (Waring et al., 1945; Forssman, 1945; Williams and Henry, 1947; Luder and Burnett, 1954; Ellborg and Forssman, 1955; Kaplan et al, 1959; Schoen, 1960; Lobeck et al., 1963; Schrager et al., 1976), particularly in the neonate and infant. Therefore, frequently symptoms are considered to be caused by infection and many children with NDI are examined thoroughly for signs of bacterial, viral or parasitic disease. It is not known how many cases remained undiagnosed during infancy and how many children have died without their disease being suspected. Seizures (Schoen, 1960; Schultz and Lines, 1975, Kanzaki et al., 1985) are rare and are most often seen during therapy, particularly if rehydration proceeds too rapidly. Obstipation is a common symptom in children with congenital NDI (Luder and Burnett, 1954; Kirman et al., 1955; Ellborg and Forssman, 1955; Schoen, 1960; Lobeck et al., 1963; ten Bensel and Peters, 1970). Nocturia and enuresis (Schoen, 1960; Carter and Goodman, 1963; Miller and Winston, 1966; Ramsey et al., 1974; Monn, 1981) are frequent complaints later in childhood and it is not uncommon for patients to get up to drink 5 to 6 times during the night because of the intense thirst.

Untreated, most patients fail to grow normally (Waring et al., 1945; Forssman, 1945; Williams and Henry, 1947; Ellborg and Forssman, 1955; Hillman et al., 1958; Kaplan et al., 1959; Schoen, 1960;

Vest et al., 1963) and some develop mental retardation (Forssman, 1955; Kirman et al., 1955;). Growth retardation is assumed to be directly related to polyuria and polydipsia (Hillman et al., 1958; Vest et al., 1963). Excessive fluid intake provokes anorexia and vomiting, which leads to malnutrition. Mental retardation, which can run the gamut from minor memory deficits to imbecility (Forssman, 1955), may be secondary to cerebral bleeding and brain damage caused by severe brain dehydration and hyperelectrolytemia occuring early in infancy (Macaulay and Watson, 1967; Kanzaki et al.,1985). Besides organic brain alterations, the psychological development of these children is influenced by a permanent craving for water and the urge for frequent voiding, which competes with playing and learning (Hillman et al., 1958). Therefore, many NDI patients are characterized by hyperactivity, distractibility, short attention span and restlessness.
Long-lasting states of polyuria may favor the development of a megacystis (Shapiro et al., 1978) and , more rarely, hydroureter and hydronephrosis (Silverstein and Tobian, 1961; Carter and Goodman, 1963; Vest et al., 1963; ten Bensel and Peters, 1970), which can mimic lower urinary tract obstruction.

1.1.3 DIAGNOSIS

The family history and the observation of polyuria in a dehydrated infant will usually provide all evidence necessary for presuming the diagnosis in an affected infant. To confirm the presence of polyuria and to distinguish the nephrogenic form of diabetes insipidus from the central form, a vasopressin test is performed by intranasal application of 1-desamino-8-D-arginine vasopressin (DDAVP) (Aronson and Svenningsen, 1974; Monnens et al., 1981; Kaplan, 1987) which is a synthetic analogue of the natural hormone and is characterized by a high and prolonged antidiuretic effect (Richardson and Robinson, 1985). In NDI patients there is, after DDAVP, no increase in urine osmolality, (Usberti et al., 1980; Monnens et al., 1984) which remains below 200 mosm/kg H_2O (normal

>805 mosm/kg H_2O), and no reduction in urine volume or in free water clearance (C_{H2O}).

It is still controversial as to whether the urinary excretion of cyclic 3',5'-adenosine monophosphate (c-AMP) can be used for differentiating vasopressin responders from non-responders. Vasopressin is considered to exert its biological effect on the kidney through the activation of the membrane-bound adenylate cyclase, which in turn converts adenosine triphosphate (ATP) to c-AMP. A defect in this mechanism has been suggested to be responsible for NDI (for detailed pathophysiological aspects see 1.2). Several investigators have observed no increase in c-AMP excretion after vasopressin infusion in NDI patients (Bell et al., 1974; Usberti et al., 1980). In these studies basal c-AMP excretion in NDI patients was found to be either elevated (Usberti et al., 1980) or significantly reduced (Bell et al., 1974) compared to control subjects. Recently Ohzeki (1985) divided NDI in two categories with respect to c-AMP response, one type with and one type without an elevation in c-AMP excretion in response to vasopressin. A major difficulty in studying NDI is that urinary c-AMP excretion after vasopressin administration is an insensitive indicator of the renal action of the hormone: in a study with ADH-deficient Brattleboro rats Bia et al. (1979) demonstrated that physiological doses of exogenous vasopressin and DDAVP increase the in situ renal papillary content of c-AMP; however, this increase could not be detected in plasma or urine. These data support the concept that the physiological action of ADH is mediated, at least in part, through c-AMP and demonstrate further that changes in c-AMP following vasopressin administration can best be detected in vivo by measurement of in situ renal papillary c-AMP content.

Plasma vasopressin levels are normal or slightly increased in affected children (Holliday et al., 1963; Gorden et al., 1971).

Other laboratory findings have been described in association with NDI, which are mainly the consequence of chronic dehydration and hyperelectrolytaemia. Glomerular filtration rate (GFR) is often slightly reduced (Cutler et al., 1962) the renal plasma flow even more, thus elevating the filtration fraction (Williams and Henry,

1947; Gorden et al., 1971). In severe dehydration states, serum urea and creatinine can be increased (Luder and Burnett, 1954; Kaplan et al., 1959; Miller and Winston, 1966; ten Bensel and Peters, 1970).

Dembowski et al.(1973) reported plasma renin to be elevated in NDI patients. The sodium chloride content of sweat is elevated in untreated cases but returns to normal after saluretic treatment (Gautier and Prader, 1956; Lobeck et al., 1963; Plöchl and Stur, 1965; Reimold, 1967), and the same was observed for saliva (Gautier and Prader, 1956). Hyperuricemia has been observed in seven adults with familial NDI (Cutler et al., 1962; Gordon et al., 1971), two of whom developed clinical gout. Urinary clearances of uric acid were reduced, while glomerular filtration rates were normal. This suggested that in adult patients, the disease may be accompanied by a renal tubular defect in uric acid excretion. Since children with familial NDI had normal serum uric acid concentrations, Gordon and co-workers (1971) proposed that such a defect might be an acquired alteration of long-lasting NDI.

With respect to other tubular function defects in NDI, reports are inconsistent. Hyperaminoaciduria and decreased renal acidification (Kaplan et al., 1959) have been occasionally described with NDI. However, in the absence of dehydration or hydronephrosis, amino-acid reabsorption rate and urinary acidification were found to be normal, as were the glucose reabsorption rate and the phosphate excretion (Williams and Henry, 1947; West and Kramer, 1955; Schoen, 1960; Miller and Winston, 1966; Schultz and Lines, 1975; Monn, 1981; Monnens et al., 1984). Perry et al.(1967) reported NDI associated with cystathioninuria.

The macroscopic anatomy of the kidney is normal (Kirman et al., 1955). Biopsy specimens of NDI patients do not show any specific tubular alterations by light microscopy (Abelson, 1968; Dembowski et al., 1973). Dembowski and co-workers (1973) observed hypertrophy of the juxtaglomerular apparatus, and Abelson (1968) found that glomeruli were hypercellular and that glomerular hyalinization was sometimes present. By electron microscopy remarkable changes were found in both proximal and distal tubules, mainly in the mitochon-

dria, which contained concentric ringed structures and myelin figures, suggesting an abnormality in mitochondrial membrane lipids (Abelson, 1968). However, these results may not be specific and have to be confirmed. Microdissection of autopsy specimens from children with the disease revealed shortening of proximal convoluted tubules (Darmady et al., 1964). It was suggested that those very short proximal tubular segments in NDI might be unable to reabsorb enough water to prevent the distal tubule from becoming overburdened with hypotonic filtrate.

The primary congenital form of NDI has to be differentiated from the secondary or acquired form, which is much more common than the congenital one, but is rarely as severe. The causes are listed in Table 1 (Relman and Schwartz, 1958; Broberger et al., 1960; Epstein, 1960; Bennett, 1970; Baum et al., 1974, Mooney et al., 1975; Singer and Forrest, 1976).

Table 1.1: Causes of acquired or secondary Nephrogenic Diabetes Insipidus.

Drug induced
- Lithium
- Tetracyclines

Analgesic nephropathy
Sickle cell anemia
Hypokalemia
Hypercalcemia
Obstructive uropathy
- posterior urethral valves

Juvenile nephronophtysis
Renal dysplasia
Chronic pyelonephritis
Chronic uremic nephropathy
Amyloidosis
Sarcoidosis

Prognosis of NDI is largely dependent on early recognition, prevention of severe dehydration and adequate nutrition during early childhood.

1.1.4 GENETICS

Most family studies have indicated that NDI is transmitted as an X-linked recessive trait and there is reason to believe that only one gene is involved (Forssman, 1945; Williams and Henry, 1947; Walker and Rance, 1954; West and Kramer, 1955; Carter and Simpkiss, 1956; Bode and Crawford, 1969). This is supported by the observations that male-to-male transmission does not occur and that the disorder is transmitted by females and fully expressed in their male offspring.

Most of the female carriers of the disease are clinically asymptomatic, although mild polydipsia and polyuria have been reported in a few women whose son had the disease (West and Kramer, 1955; Carter and Simpkiss, 1956).

Doubts about whether X-linked inheritance is the only type of transmission in NDI raised when several investigators (Robinson and Kaplan, 1960; Schreiner et al., 1978) described the complete clinical picture of the disease to occur in females. They postulated an autosomal dominant inheritance with almost complete penetrance in the male and with reduced penetrance in the female. This was supported by Cannon (1955), who reported male-to-male transmission on six occasions. However, critical review of these pedigrees (Mc Kusick, 1971) revealed that the original proposal of X-linked transmission is probably correct and might even fit for Cannon's pedigree, since all six cases of male-to-male transmission occurred in early generations, for which one had to rely on history data for the diagnosis as well as the exclusion of consanguinity. Recently, we were able to confirm X-linked inheritance by demonstrating close genetic linkage between the NDI gene and several X chromosomal DNA markers (Knoers et al, 1987, 1988a, 1988b, 1989), which allowed precise localization of the disease gene to the sub-

telomeric region of the human X chromosome long arm, at band Xq28. The localization of the NDI gene and its use for reliable carrier detection and early (prenatal) diagnosis will be discussed in detail later (chapter 2, 3, and 4).
The prevalence of NDI is not exactly known, but the disorder is assumed to be quite rare, since even in large pediatric and nephrology clinics it is observed infrequently (Kaplan, 1987). Isolated cases of the disease have encountered, with no evidence of affected relatives, suggesting new mutations (Ellborg and Forssman, 1955; Gautier and Prader, 1956; Feigin et al., 1970). Bode and Crawford (1969) postulated that the rate of new mutations might be very low, and suggested that almost all patients with NDI in North America were descendents of the 'Ulster Scottsmen' who arrived in Nova Scotia aboard the ship 'Hopewell' in the 18th century.

1.1.5 TREATMENT

The treatment of NDI has been a problem since the original description of the disorder. Replacement of urinary water losses by adequate supply of fluid is the most important component of therapy. This is easily achieved by oral intake in children and adults, but often requires parenteral supplementation in infants. One approach to reduce urine output is provision of a low solute diet to reduce the osmolar load and decrease the obligatory water excretion (Hillman et al., 1958). Initially, a diet low in sodium (1 mmol/kg/day) as well as protein (2 g/kg/day) was recommended (Hillman et al., 1958; Brodehl, 1981). However, severe limitations of dietary protein may introduce serious deficiencies. Therefore, it seems preferable to prescribe a dietary restriction of sodium only.
Treatment of NDI could be dramatically improved when Crawford and Kennedy (1959) and, independently, Reerink and co-workers (1959) introduced the use of thiazide diuretics for treatment of the disorder. They demonstrated that chronic administration of chlorothiazide could result in an increase in urine osmolality and a decrease in urine volume as well as thirst. Several hypotheses for

the mechanism(s) underlying the paradoxical antidiuretic effects of these thiazides in NDI have been reported. The observation that concomitant salt administration could prevent the antidiuretic effect of these drugs (v.d. Korst, 1965), implied that the efficiency of these agents, which inhibit sodium reabsorption in the early distal tubule, is related to the induction of a negative salt balance. The resultant decrease in extracellular fluid volume with a normal serum sodium and, possibly, the reduction in glomerular filtration rate (GFR) could serve to enhance sodium and fluid reabsorption in the proximal tubule (Crawford and Kennedy, 1959; Earley and Orloff, 1962). In this way the sodium and water delivery to the distal tubules would be diminished and antidiuresis might result unrelated to the presence or effect of vasopressin. This assumption was favoured by studies demonstrating that other saluretics, such as ethacrynic acid (Brown et al., 1966; Ramos et al., 1967), furosemide (Reimold, 1967; von Brenndorf and Hagge, 1973) and spironolactones (Kowarski et al., 1966) were also effective in producing antidiuresis. However, Shirley et al. (1982) demonstrated that during chronic hydrochlorothiazide administration Brattleboro rats, which have hereditary diabetes insipidus due to lack of ADH, showed only a small reduction in the volume of fluid delivered to the more distal nephron segments. They suggested that the raised papillary osmolality, by enhancing water reabsorption at sites beyond the proximal tubule, makes a greater contribution to the antidiuresis. The thiazide induced increase in medullary osmolality was not explained. It was first postulated by Brown et al. (1969) that the antidiuresis induced by saluretics might be mediated by renin and angiotensin. Sodium depletion stimulates renin production, which causes increased levels of angiotensin, and this in turn stimulates aldosterone release and sodium reabsorption. This hypothesis was supported by the observation of significantly increased plasma renin levels in patients with diabetes insipidus (Brown et al., 1969) and NDI (Dembowski et al., 1973) which were treated with saluretics. In addition, it was shown that angiotensin can indeed produce a marked antidiuresis in NDI (Orr and Filipich, 1967). Whatever the mechanism, administration of thiazide

diuretics combined with a low sodium diet, will generally reduce polyuria by about 20-50 % of the original value (Crawford et al., 1960). Since these drugs induce kaliuresis also (Morgan and Davidson, 1980), treatment should be associated with oral supplementation of potassium salt in most cases.

Several publications, but not all (Monnens et al., 1984), have indicated elevated prostaglandin E_2 excretion in NDI patients (Fichman et al., 1976; Usberti et al., 1980; Blachar et al., 1980). It is not clear whether increased prostaglandin activity represents part of a primary defect in NDI or whether it is a secondary phenomenon, for instance due to elevated ADH levels. It is generally accepted that ADH stimulates medullary prostaglandin E_2 production (reviewed by Beck and Dunn, 1981), but the significance of this regulation is still unclear. Whatever the cause, the observation of increased prostaglandin E_2 excretion in NDI formed the basis for treatment of the disease with prostaglandin synthesis inhibitors, such as indomethacin (Fichman et al., 1976; Usberti et al., 1980; Blachar et al., 1980; Monnens et al., 1984; Libber et al., 1986; Rasher et al., 1987), iboprufen (Fichman et al., 1978) and acetylsalicylic acid (Monn, 1981). Administration of these agents appeared to have a beneficial effect on urinary output in NDI, especially when combined with hydrochlorothiazide (Blachar et al., 1980; Monnens et al., 1984; Rasher et al., 1987). However, the mechanisms by which prostaglandin synthesis inhibitors cause a reduction in urine volume in NDI are not exactly known. Despite many contradictions in the literature there is evidence that prostaglandins antagonize the ADH-sensitive adenylate cyclase in the collecting tubule (reviewed by Beck and Dunn, 1981) and that, on the other hand, inhibitors of prostaglandin synthesis potentiate the effect of ADH on collecting duct cells. Inhibitors of prostaglandin synthesis enhanced ADH-induced water permeability in the toad bladder (Albert and Handler, 1974; Ray and Morgan, 1981) and rat medullary collecting duct (Jackson et al., 1980a) and increased maximum urine osmolality in dogs (Anderson et al., 1975) and humans (Berl et al., 1977) given vasopressin. Lum et al.(1977) demonstrated that administration of indomethacin to rats enhanced

accumulation of medullary cyclic AMP in reponse to a submaximal dose of ADH. They were careful to establish that the effect of indomethacin was not due to an inhibition of phospodiesterase, that degrades c-AMP to its 5' monophosphate counterpart. This is an important observation, since indomethacin does inhibit phosphodiesterase in vivo (Newcombe et al., 1974) and in vitro (Berl et al., 1977). Since c-AMP mediates the hydroosmotic effect of ADH, it was postulated that the increase in tissue c-AMP level could be responsible for the enhanced physiologic response to the hormone. However, the presence of ADH is not essential for the antidiuretic effect of prostaglandin synthesis inhibitors, since it was demonstrated that these agents also reduced urine output in Brattleboro rats, known to lack ADH (Stoff et al., 1981) and in humans with central diabetes insipidus (Fichman et al, 1978). Alternatively, mechanisms independent of ADH might underly the water reabsorption and decrease in urine volume observed after administration of prostaglandin synthetase inhibitors. In animals treated with indomethacin increased papillary solute concentrations, acting as a driving force for enhanced water reabsorption, have been demonstrated (Ganguli et al., 1977; Passmore et al., 1980; Stoff et al., 1981). The increased papillary tonicity can be ascribed either to a reduction in papillary solute wash-out resulting from decreased blood flow (Passmore et al., 1980; Ganguli et al., 1981; Stokes, 1981) or to an increase of sodium chloride transport in the thick ascending limb and collecting ducts (Stokes, 1981; Stoff et al., 1981; Libber et al., 1986).

Finally, a reduction in glomerular filtration rate (GFR) might contribute to the antidiuretic action of indomethacin and other prostaglandin synthesis inhibitors (Monnens et al., 1984; Rasher et al., 1987). However, a significant fall in GFR response to prostaglandin synthesis inhibitors has not been observed in all patients with NDI (Libber et al., 1986).

Whatever the explanation, indomethacin and other prostaglandin synthetase inhibitors have proven to be an important gain in the treatment of NDI, especially in young children. However, numerous reports of possible systemic and renal adverse effects due to the

treatment with prostaglandin synthesis inhibitors have appeared in the literature (Walshe and Venuto, 1979; Tan et al., 1979). Dyspepsia (Boardman and Hart, 1967) and peptic ulcer are known side effects of chronic administration of indomethacin, aspirin or ibuprofen. Vomiting, abdominal distention, melaena, and necrotising enterocolitis have been observed in relation with indomethacin therapy (Harinck et al., 1977). Tan et al. (1979) reported the occurrence of acute renal failure during indomethacin therapy in a patient with chronic pyelonephritis.
An alternative therapeutic approach was recently proposed by Alon and Chan (1985). In view of the previously reported efficacy of the potassium sparing diuretic amiloride as treatment for lithium-induced NDI in rats (Feuerstein et al., 1981) and humans (Battle et al., 1983), they tested the combination amiloride-hydrochlorothiazide in 2 NDI patients, with encouraging results. This alternative therapy regimen will be discussed in detail in chapter 7.

1.2 NEPHROGENIC DIABETES INSIPIDUS: PATHOPHYSIOLOGY

1.2.1 INTRODUCTION

In order to understand the pathophysiological mechanism(s) involved in NDI, it is necessary to consider the physiological effects of vasopressin (antidiuretic hormone, ADH) on the kidney. Vasopressin subserves a number of different functions in the mammalian kidney. These include:

(1) Modulation of water (and solute) transport (Hebert and Andreoli, 1984; Tomita et al., 1986; Abramow et al., 1987; Hebert et al., 1987; Sands et al., 1987; Sands and Knepper, 1987).
(2) Vasoconstriction (Andrews and Brenner, 1981; Kreisberg, 1983; Schlondorf, 1987) (see 1.2.5).
(3) Stimulation of prostaglandin synthesis (Kirschenbaum et al., 1982; Ardaillou et al., 1985; Wuthrich and Valloton, 1986) (see 1.1.5).

(4) Inhibition of renin secretion.

It is well established that vasopressin inhibits renin secretion in a variety of species including man (Keeton and Campbell, 1981; Share, 1988). The inhibition of renin secretion is related to the dose of vasopressin and can be produced by elevations in plasma vasopressin within the physiological range. The mechanism of inhibition of renin secretion by vasopressin is not well understood. Possible actions that have been suggested to result in inhibition of renin secretion include volume expansion, vasoconstriction, altered renal handling of sodium, decreased renal sympathetic nerve activity, release of atrial natriuretic peptide from the heart or a direct action of vasopressin in the juxtaglomerular apparatus (Keeton and Campbell, 1981; Itoh et al., 1987; Share, 1988).

Each of these functions is mediated by either one of two receptor subtypes, termed V_1 and V_2 receptors (Michell et al., 1979). These distinct receptor subtypes have been recognized on the basis of both functional (Michell et al., 1979; Jard, 1988) and pharmacological criteria (Stassen et al., 1982; Jard, 1988; Kinter et al., 1988; Liard, 1988; Thibonnier, 1988). The V_1 receptor is functionally connected to a phosphoinositide specific phospholipase and to Ca^{2+} mobilization, while the V_2 receptor is coupled to the membrane bound adenylate cyclase. In addition, V_1 and V_2 receptors can be distinguished by their molecular sizes (Crause et al., 1984). For the most part, the V_2 receptor subserves epithelial functions, such as water and solute transport, while the V_1 receptor subserves funtions on cells of mesenchymical origin, such as vasoconstriction and stimulation of prostaglandin synthesis. Recently, the existence of both V_1 and V_2 receptors in the principal cells of the rabbit cortical collecting tubule was demonstrated (Burnatowska-Hledin and Spielman, 1989). The physiological significance of this dual receptor system is not exactly known. In amphibian bladders interactions between the V_1 receptor-mediated phosphoinositide pathway and the V_2 receptor-mediated c-AMP pathway have been described (Schlondorff and Levine, 1985). These studies demonstrate

that direct activation of proteine kinase C (which is an intermediate in the phosphoinositide pathway) by phorbol esters suppresses ADH induced water flow. Based on the assumption that V_1 and V_2 receptors exist in the same renal epithelial cells, Schlondorff and Levine (1985) postulated that V_1 receptor occupancy serves to down-regulate the V_2 receptor response. However, it is not known if these observations in amphibian bladders also apply to the mammalian kidney. There is strong evidence for the existence of extrarenal V_2 receptors (Chapter 5 and 6; Kobrinski et al., 1985; Bichet et al., 1988). However, the localization of these vasopressin receptors remains to be elucidated. Extrarenal V_1 receptors have been identified on liver (Tolbert et al., 1980; Fishman et al., 1987), vascular smooth muscle cells (Fox et al., 1987; Stassen et al., 1987) and on platelets (Thomas et al., 1983; DiTullio et al., 1985; Vittet et al., 1986; Inaba et al., 1988).
In this chapter the first function of ADH , the modulation of water (and solute) transport, will be discussed in detail. This is the all-important action by which ADH controls the volume and osmolality of the urine, in order to maintain water balance. Before discussing this action of vasopressin (1.2.4), two processes that are involved in the regulation of water excretion by ADH, are described: (1) ADH release (1.2.2) and (2) The build-up and maintenance of a cortico-papillary interstitial osmotic gradient by the renal countercurrent system (1.2.3). In section 1.2.5 a brief description of the extrarenal effects of ADH will be given. Section 1.2.6 describes an animal model that phenotypically closely resembles human hereditary NDI. Finally, in section 1.2.7., the mechanisms are discussed, which might underly the unresponsiveness to vasopressin in NDI.

1.2.2 ANTIDIURETIC HORMONE RELEASE

Arginine vasopressin (AVP) or antidiuretic hormone (ADH) is a cyclic nonapeptide, produced in the supraoptic and paraventricular nuclei in the hypothalamus, transported along the axons of the

supraopticoneurohypophyseal tract, and stored in the posterior pituitary (Scharrer and Scharrer, 1954). Under normal circumstances ADH release is primarily regulated by changes in plasma osmolality (Robertson, 1987), which are monitored by osmoreceptive mechanisms. These osmoreceptors are multiple in nature and are localized both centrally and peripherally (Sladek and Armstrong, 1985). The system regulating the osmolality-dependent release of ADH is very sensitive and reacts to changes in plasma osmolality as small as 1 % (Dunn et al., 1973).

Vasopressin release, however, can also occur in the absence of changes in plasma osmolality (Schrier et al., 1979). The nonosmotic stimuli for vasopressin secretion generally are related to changes in either total blood volume or in the distribution of extracellular fluid, such as in shock, blood loss due to trauma, cardiac failure, liver disease, and adrenal insufficiency. In addition, physical pain, emotional stress and certain drugs (i.e. nicotine) influence the release of ADH. The nonosmotic regulation of ADH is modulated mainly by baroreceptors within the great vessels in the thorax (Gauer and Henry, 1963), stretch receptors in the atria of the heart (Henry et al., 1956), and possibly also by carotid sinus chemoreceptors (Share and Levy, 1966) (see also review in Share, 1988). This nonosmotic pathway is less sensitive than the osmotic one (Dunn et al., 1973). Recently, interest has been focussed on possible central mediators of vasopressin release that could serve to affect the release of the hormone in response to osmotic as well as nonosmotic stimuli (Sklar and Schrier, 1983), for instance biogenic amines, prostaglandins, and angiotensin II. In addition, atrial natriuretic factor (ANF) was found to have a central inhibitory effect on the basal secretory rate of vasopressin in sheep (Lee et al., 1987) and in the rat (Poole et al., 1987).

1.2.3 BUILD-UP AND MAINTENANCE OF A CORTICO-PAPILLARY INTERSTITIAL OSMOTIC GRADIENT.

The existence of an osmotic gradient in the renal medullary inter-

stitium provides the driving force for water reabsorption from the ADH-responsive portions of the distal nephron (the collecting duct system). The build up and maintenance of such a gradient is dependent on the normal functioning of the so-called renal countercurrent system (Kuhn and Ryffel, 1942). Following a short description of the functional unit of the kidney, the principles of this countercurrent mechanism will be discussed in this section.

The functional unit of the kidney is the nephron. There are at least two types of nephron: the outer cortical (or superficial) and the juxtamedullary. These and their relationship to the major zones of the kidney are shown in Fig. 1.

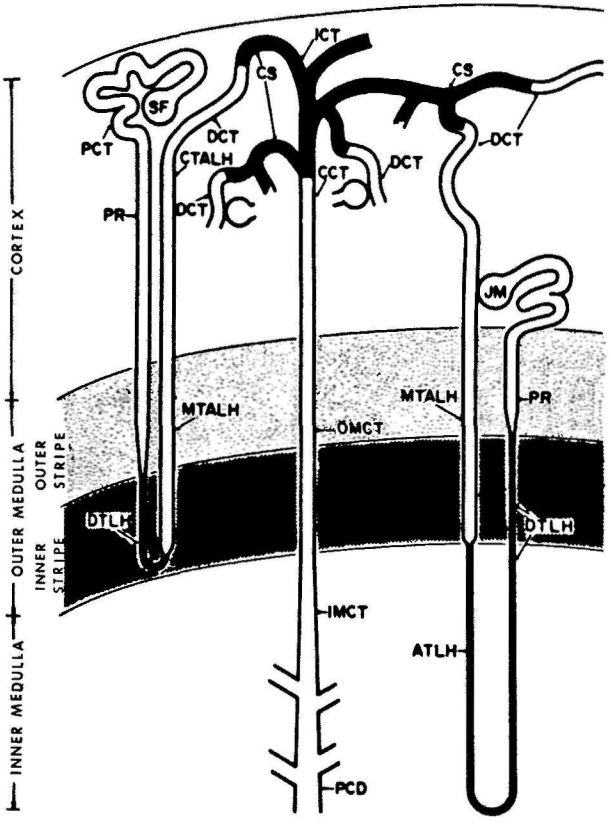

Fig.1.1 :
Two nepron populations, superficial, (SF) and juxtamedullary (JM) are depicted. The major nephron segments are labeled as follows: PCT = proximal convoluted tubule; PR = pars recta; DTLH = descending thin limb of Henle; ATLH = ascending thin limb of Henle; MTALH = medullary thick ascending limb of Henle; CTALH = cortical thick ascending limb of Henle; DCT = distal convoluted tubule; CS = connecting segment; CCT = cortical collecting tubule; OMCT = outer medullary collecting tubule; IMCT = inner medullary collecting tubule; PCD = papillary collecting duct.
(Taken from Jacobson, H.R. - Functional segmentation of the mammalian nephron. Am.J. Physiol. 241, 1981, F203-F218, with permission)

Each nephron is build up of a glomerulus, surrounded by Bowman's capsule, then successively a proximal tubule, the straight portion (pars recta) of which reaches through the outer strip of the outer medulla, a descending limb of Henle's loop, a thin ascending limb of Henle's loop (in the juxtamedullary nephrons only), a thick ascending limb of Henle's loop, reaching through the outer medulla into the cortex, a distal tubule and a collecting tubule. A juxtamedullary nephron is distinguished from an outer cortical nephron by the following characteristics: it has a long loop of Henle which turns within the inner medulla; and its postglomerular blood supply gives rise to the vasa recta, which course through the outer and inner medulla (Fig. 2).

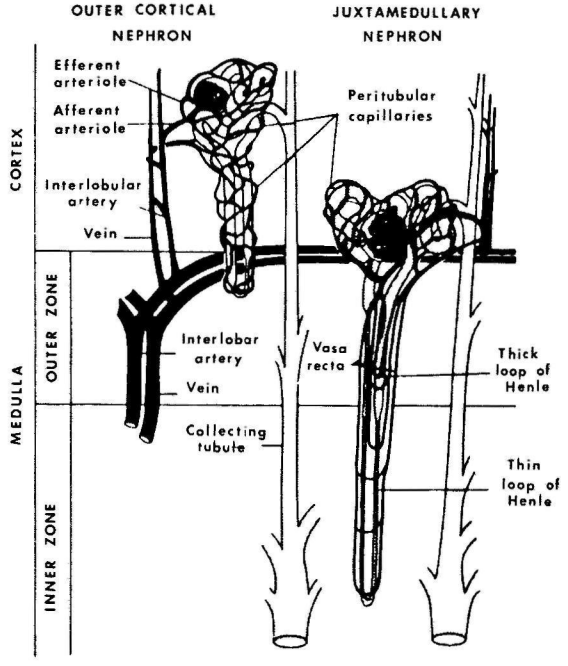

Fig. 1.2:
Comparison of the anatomy and blood supplies of outer cortical and juxtamedullary nephrons. (Adapted from Pitts, R.F. - Physiology of the kidney and body fluids, 3d ed., 1974, Year Book Medical Publishers, Chicago)

These anatomic features have important functional consequences, as will be seen later.

Urine is concentrated with relative little expenditure of metabolic energy by the renal countercurrent mechanism, a complex interaction between the loops of Henle, the medullary interstitium and the collecting tubule. The two fundamental processes of the countercurrent mechanism, countercurrent multiplication (a) and countercurrent exchange (b) will be delineated in the following.

(a) Countercurrent multiplication

In 1942 the functional significance of the loop of Henle was proposed when Kuhn and Ryffel developed the concept of countercurrent multiplication for urine concentration. The core of this hypothesis is that a small difference in osmotic gradient (the so-called single effect) at any point between fluid in opposite directions in two parallel tubes, connected in a hairpin manner, can be multiplied many times along the length of these tubes. Support for this hypothesis was subsequently given by Hargitay and Kuhn (1951), Wirz et al. (1951), and Gottschalk and Mylle (1959), who stated that the requirements for countercurrent multiplication are readily satisfied in the loop of Henle. The ascending and descending limbs of Henle's loop exhibit a countercurrent flow and have a striking difference in membrane permeabilities that are essential for this process. The descending limb is highly permeable to water and to a lesser degree to solutes (Kokko, 1970). Both the thin and the thick ascending limbs are water-impermeable. The thick ascending limb possesses an active Cl^- transport system (Burg and Green, 1973; Rocha and Kokko, 1973), which provides an initial step in countercurrent multiplication. The principle of this process is shown in Fig. 3. Initially, the fluid in the descending and ascending limb and in the interstitium is isoosmotic to plasma, similar to that delivered from the proximal tubule (a). The active transport of NaCl in the thick ascending limb dilutes the tubule fluid in this segment and concentrates the medullary interstitium. Then the tubule fluid in the descending limb equilibrates to the hypertonic medullary interstitium (b). As urine passes through the tubules,

and NaCl transport in the ascending limb continues, the initial step is multiplied by the countercurrent flow arrangement of the loops of Henle (c - f).

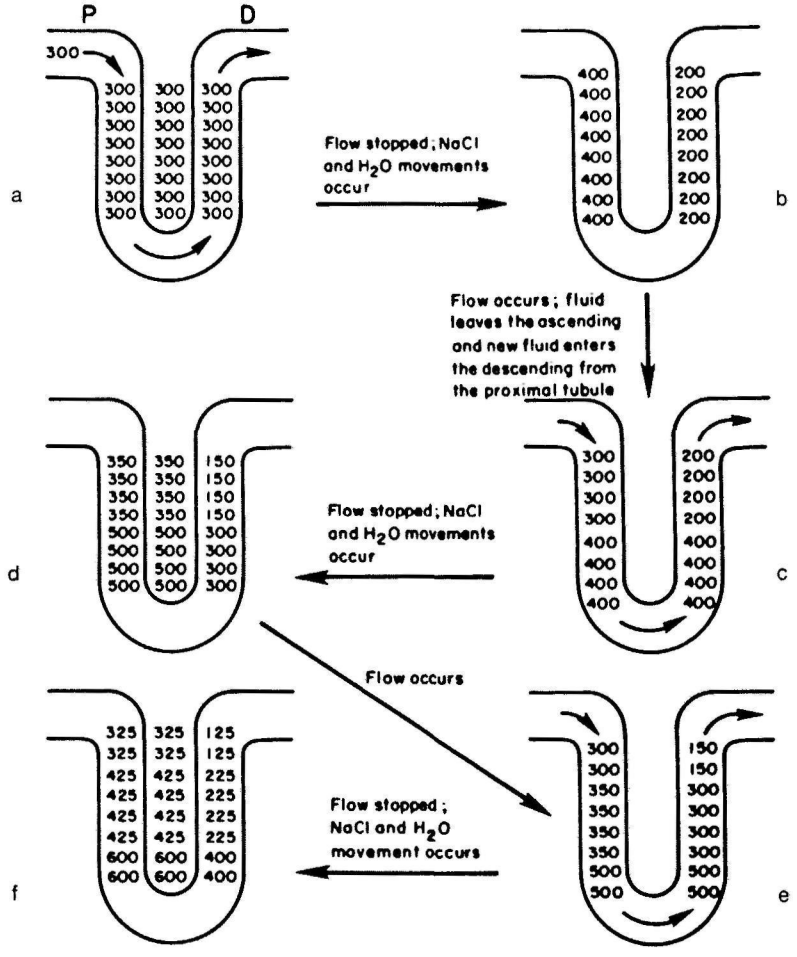

Fig 1.3:
Countercurrent multiplication system in the loop of Henle. (Adapted from Pitts, R.F. - Physiology of the kidney and body fluids, 3d ed., 1974, Year Book Medical Publishers, Chicago)

Ultimately, a high osmolar concentration difference between the cortico-medullary junction and the hairpin loop at the tip of the papilla is generated. This form of countercurrent multiplication, which depends on active Cl^- transport, occurs primarily in the thick ascending limbs of Henle's loop, which are located in the outer medulla (short loops).

However, in the thin ascending limbs, located in the inner medulla (long loops), where the greatest rise in osmolality occurs, an active Cl^- transport process has not been identified (Imai and Kokko, 1974; Jamison, 1987). Considering this, Kokko and Rector

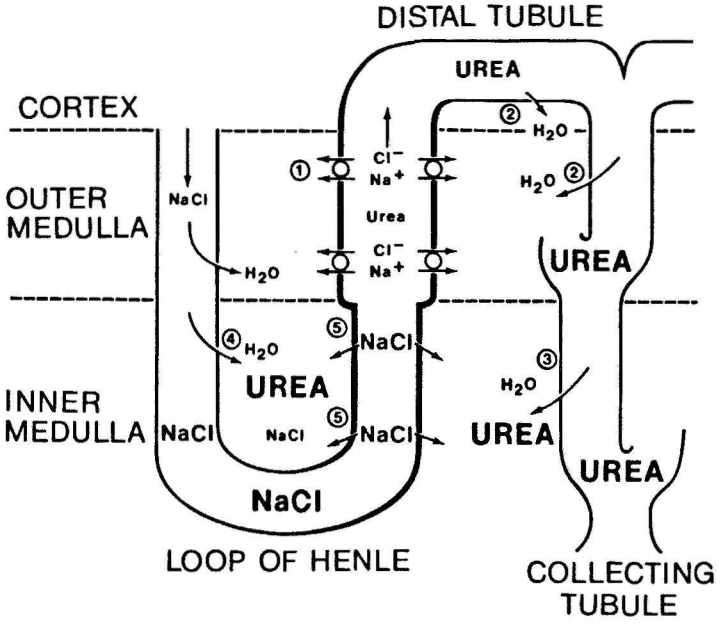

Fig. 1.4:
Schematic representation of the passive urinary concentration mechanism, according to the model of Stephenson (1972) and Kokko and Rector (1972). (Taken from Jamison, R.L., Maffly, R.H. - The urinary concentrating mechanism. N.Engl.J.Med. 295, 1976, 1059-1066, with permission)

(1972) and simultaneously Stephenson (1972) proposed a model which provides two spatially distinct sites for countercurrent multiplication: an active step in the outer medulla and a passive step in the inner medulla, the latter involving urea recycling. In this model, active transport of sodium chloride by the thick ascending limb creates the osmotic gradients that allow water reabsorption to occur in the collecting tubule. This results in a concentrated solution of urea, which is delivered to the inner medullary collecting duct, where, in the presence of ADH (see 1.2.4), urea is reabsorbed and trapped in the inner medulla. High urea concentrations extract water from the descending limb and provide for NaCl concentration gradients across a salt-permeable water-impermeable thin ascending limb (Fig. 4). Reabsorption of NaCl thus provides the passive step in countercurrent multiplication. Many alternative models were put forward (review in Jamison, 1987).

(b) Countercurrent exchange (Berliner et al., 1958; Fig. 5)
This process occurs in the vasa recta. Like the loop of Henle, the vasa recta form a counterflow system, in which blood flows in opposite directions in descending and ascending segments. In the descending vasa recta water leaves the blood and solute enters. A progressive rise in osmolality in the descending blood ensues, reaching the maximum at the hairpin turn. As blood ascends again, it gains water and loses salt to the progressively less hypertonic medullary interstitium. This arrangement minimizes the degree to which blood flow through the medulla decreases the hyperosmolality of that area. Thus, countercurrent exchange is a crucial factor in the maintenance of a corticopapillary osmotic gradient. A relatively low blood flow through the medulla is essential for maintaining the hyperosmolality of the medullary interstitium. In this regard, it is interesting to note that, at least in the rat, ADH decreases inner medullary blood flow (Zimmerhackl et al., 1985). This effect of ADH is mediated, at least in part, by the V_1 receptor, since an antagonist of the V_1 action of ADH prevented the vasopressin-induced decrease in medullary blood flow. Recently, Kiberd and co-

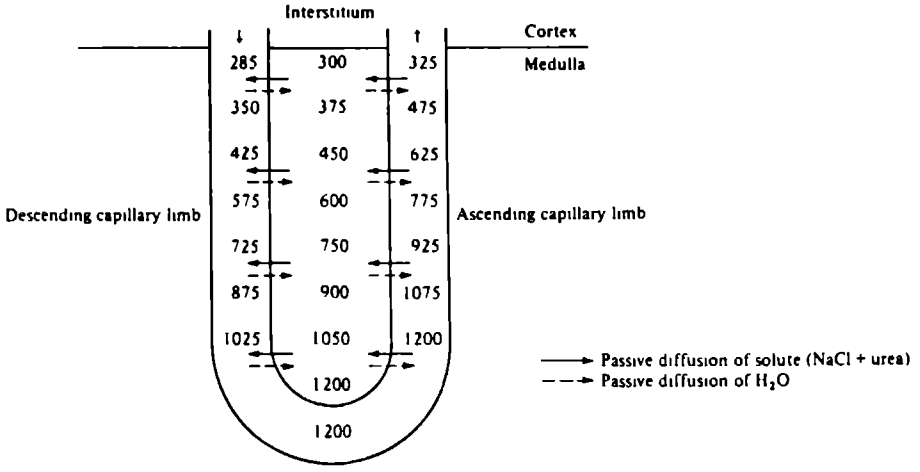

Fig. 1.5:
Principle of countercurrent exchange in the vasa recta capillaries. In the descending capillary limb, solute enters and water leaves the capillary down concentration gradients, tending to reduce interstitial osmolality. These processes are reversed in the ascending limb, thereby preserving the interstitial gradient. (Adapted from Pitts, R.F. - Physiology of the kidney and body fluids, 3d ed., 1974, Year Book Medical Publishers, Chicago)

workers (1987) suggested that the ADH-induced decrease in inner medullary blood flow is also mediated in part by its antidiuretic (V_2 receptor) action. Using fluorescence videomicroscopy, they demonstrated that during infusion of a V_2 inhibitor with ADH or the inhibitor alone, medullary blood flow did not fall, but revealed instead a tendency to increase with time. The V_2 inhibitor did not inhibit the pressor effect of vasopressin.

1.2.4 MODULATION OF WATER AND SOLUTE TRANSPORT BY VASOPRESSIN.

1. Modulation of water transport.

The regulation of water absorption across the collecting duct system, defined as the late distal tubule and the collecting ducts, is the most important function through which ADH determines the concentration of urine (DeSousa, 1984). When ADH levels are unde-

tectable, these portions of the nephron are relatively impermeable, and hypotonic urine, leaving the ascending limb of Henle's loop, is excreted. In the presence of ADH, these portions of the nephron become more permeable to water, and the tubular fluid is concentrated as it passes down the collecting system and equilibrates with the hypertonic medullary interstitium.

Much has been learned about the mechanism by which vasopressin induces this effect. An review of the cellular events secondary to vasopressin action was recently published by Abramow et al.(1987). The initial step involves the binding of ADH to a specific receptor located at the outer surface of the basolateral membrane of responsive epithelial cells. The vasopressin-receptor complex activates the enzyme adenylate cyclase, which catalyzes the formation of cyclic adenosine monophospate (c-AMP) from adenosine triphosphate (ATP). c-AMP, in turn, activates a protein kinase which initiates a sequence of events ultimately resulting in increased permeability of the apical (luminal) membrane.

In the following, the steps resulting in increased production of c-AMP, the terminal events at the luminal membrane and finally, the intermediate steps triggered by c-AMP, will be discussed in more detail.

Events leading to increased production of c-AMP.

Receptors

It is generally accepted that the renal vasopressin receptors, which mediate the antidiuretic action of the hormone, are functionally coupled to the membrane bound adenylate cyclase, an enzyme catalyzing the formation of cyclic adenosine monophosphate (c-AMP) from its precursor adenosine triphosphate (ATP) (Orloff and Handler, 1962; Anderson and Brown, 1963; Grantham and Burg, 1966; Rajerison et al., 1974). Vasopressin-sensitive adenylate cyclase activity has been identified in several segments of the mammalian nephron (the cortical and medullary segments of the collecting duct and, in some species [rat, mouse, but not man], the ascending limb of Henle's loop) (Morel, 1981; Morel et al., 1987). Cellular adeny-

late cyclase activity was found to be proportional to the degree of hormone binding to receptor sites (Rajerison et al., 1974; Butlen et al., 1978; Hechter et al., 1978a). The renal vasopressin receptor belongs to the V_2 receptor subtype. Specific vasopressin binding sites have been characterized on renal membranes from various mammalian species, including man (Bockaert et al., 1973; Rajerison et al., 1974; Roy et al., 1975; Butlen et al., 1978; Hechter et al., 1978a, 1978b; Guillon et al., 1982). Vasopressin binds to a single class of high affinity receptors. The dissociation constant varies depending on the species from 0.4 nM (rat) to 10-20 nM (pig) (Jard, 1983). Binding is specific, time-dependent, reversible and saturable. Recently, Steiner and Philips (1988) published the binding characteristics of vasopressin receptors specifically for a basolateral membrane preparation of rat renal epithelia. Their data confirm the existence of high-affinity, highly specific V_2 receptors on these membranes. Vasopressin receptor down-regulation has been reported to occur in response to physiologically elevated levels of vasopressin (Roy et al., 1981; Steiner and Philips, 1988). The molecular structure of the renal V_2 receptor and of the effector system in renal membranes is unknown at present. The molecular size of the V_2 receptors in bovine and rat kidney membranes was determined, using the technique of radiation inactivation (target size analysis) (Crause et al., 1984). The inactivation of membrane bound renal V_2 receptors by ionizing radiation was determined by measuring the decay of the specific binding capacity for vasopressin. Molecular sizes of approximately 95 kDA (rat kidney) and 108 kDA (bovine kidney) were found. This technique gives the molecular weight (M_r) of a functional complex rather than the molecular weight of a subunit. To identify renal vasopressin receptor proteins, Fahrenholz and co-workers (1985, 1988) developed a series of photoreactive vasopressin analogues, with a photoreactive arylazido-group in position 4 or 8 of the vasopressin amino acid sequence. These arylazido analogues can be activated by flash photolysis with ultraviolet light. When activated, these ligands bind to the receptor and induce a permanent stimulation of renal vasopressin-sensitive adenylate cyclase. In photoaffinity experiments with

tritium labelled ligands, a membrane protein from bovine kidney or rat kidney medulla with an apparent molecular mass of 30,000 was preferentially labelled and to a lesser degree also a protein with of M_r 60,000. The results suggested that a 30,000 M_r protein is the binding subunit of the renal V_2 receptor. In addition, these studies provide evidence for a second subunit of the renal V_2 receptor with a M_r of about 60,000.

Guanine nucleotide binding proteins

Recent progress has been made into the mechanisms of the modulation at the transduction step between V_2 receptor occupancy and the activation of adenylate cyclase. In the last decade, it has become clear that all membrane receptors interact with effector systems to modulate the intracellular levels of a second messenger via the intermediacy of members of a family of membrane-bound guanine nucleotide binding proteins (G-proteins or N-proteins). Rodbell and coworkers (1971), whilst studying the ability of the peptide hormone glucagon to stimulate adenylate cyclase activity in hepatocytes, were the first to demonstrate a specific requirement for guanine nucleotides in hormonal function. Since these pioneering studies, a considerable number of G-proteins has been discovered, some of which have been purified and cloned (Birnbaumer et al., 1987; Lochrie and Simon, 1989). The G-proteins are heterotrimers. Their subunits are designed α, β, and γ in order of decreasing molecular mass. The α-subunit carries the nucleotide binding site. Alpha-subunits clearly differ among the members of the family. The β chains are nearly identical, whereas the γ subunits show some differences. Recent reviews (Stryer and Bourne, 1986; Spiegel, 1987; Gilman, 1987) give a detailed description of the mechanism of activation of G-proteins. Fig.6 summarizes the current view on this process. In the resting state, the G-protein exists in the holomeric form with GDP bound to the nucleotide binding site of the α-subunit. Hormone-receptor binding produces a change in receptor-G-protein interaction, allowing GTP to replace GDP on the α subunit. Release of GDP must obviously preceed the binding of GTP, because there is only one site for nucleotide binding on the α-subunit. Release of GDP

appears to be the rate limiting step in G-protein activation/inactivation. With GTP in the nucleotide binding site and in the presence of Mg^{2+} (Iyengar and Birnbaumer, 1981; Birnbaumer et al., 1985), the holomeric G-protein dissociates into an active α-subunit with bound GTP and free β/γ units. This active α-subunit is then able to modify the activity of the effector system (in this case: adenylate cyclase). Hydrolytic cleavage of the terminal phosphate from the bound GTP by intrinsic GTP-ase activity deactivates the α-subunit and in this GDP bound form is then able to reassociate with β/γ subunits to restore the inactive G-protein.

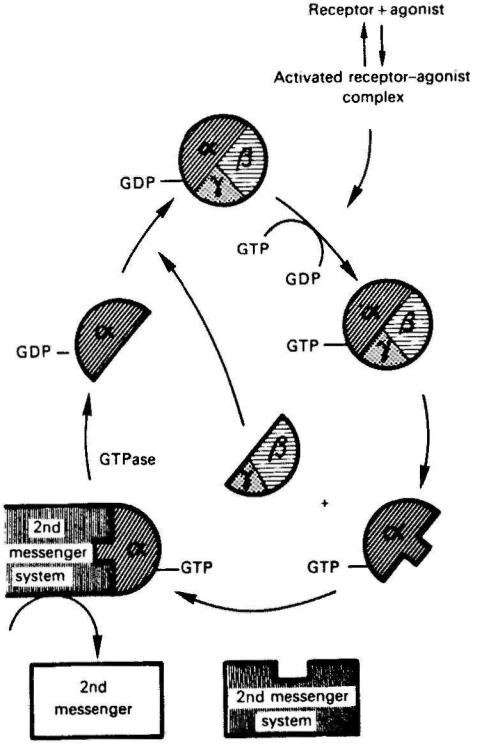

Fig. 1.6:
The role of GTP binding and hydrolysis in the activation and deactivation of a typical G-protein. (Taken from Milligan, G. - Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. Biochem.J. 225, 1988, 1-13, with permission)

Two bacterial toxins can interrupt the signal transduction cycle at specific stages. Pertussis toxin uncouples receptors from G-proteins thereby abolishing signaling (Jakobs et al., 1984), while cholera toxin inhibits GTP-ase activity which enhances signaling (Gill, 1977).

Two G-proteins are involved in the activation of adenylate cyclase activity, which either activate (G_s) or inhibit (G_i) adenylate cyclase, when the activating (R_s) or inhibiting (R_i) receptor is occupied respectively (Birnbaumer et al., 1986). Vasopressin uses the activating pathway while alpha-2-adrenergic agents, for instance, are inhibitory by occupying R_i (Edwards and Gellai, 1988). Both G-proteins have been purified and their molecular structures have been characterized (Northup et al., 1983a, 1983b; Bokoch et al., 1983; Codina et al., 1984). They are structurally very similar, albeit not identical, proteins (reviews in: Birnbaumer et al., 1986; Gillman, 1987; Spiegel, 1987). Activation of both proteins through hormone-receptor interaction involves the sequential steps of GTP-GDP exchange, magnesium addition, and dissociation of their respective α, β/γ subunits. The GTP-bound dissociated form of the α subunit combines with the adenylate cyclase, to result in either stimulation or inhibition. Besides a direct inhibitory action of the α subunit of G_i with adenylate cyclase, an indirect action of its β subunit to inhibit G_s subunit dissociation has been suggested (Katada et al., 1984a, 1984b; Birnbaumer et al., 1986). However, the exact mechanism by which G_i proteins affect adenylate cyclase remains unknown (Pingoud et al., 1988).

Receptor-independent activation of G_s and G_i can occur using non-hydrolysable guanyl nucleotide analogs or the fluoride ion (Northup et al., 1983a, 1983b; Katada et al., 1984a, 1984b; Birnbaumer et al., 1985).

Direct stimulation of adenylate cyclase, independent from receptor and G-protein has been demonstrated using forskolin (Nadler et al., 1986).

Interactions with vasopressin at the level of G-proteins have been shown for prostaglandins (Nadler et al., 1986) and bradykinin (Schuster, 1985). Both substances are generated in the kidney and

inhibit the hydroosmotic response to vasopressin. In the isolated rabbit cortical collecting tubule, exogenous PGE_2 reversibly inhibited the hydroosmotic response to vasopressin and to cholera toxin, but not the response to forskolin (Nadler et al., 1986). This sets the site of vasopressin-prostaglandin interaction at the level of the G-protein. The mechanism of action of bradykinin has been explored using a similar experimental approach (Schuster, 1985).

c-AMP breakdown

The intracellular concentration of c-AMP is determined not only by its rate of formation, but also by its rate of breakdown, which is catalyzed by a c-AMP phosphodiesterase. Inhibitors of this phosphodiesterase, such as theophylline (Orloff and Handler, 1967), aminophylline or chlorpropamide (Chaudhuri and Winer, 1970) can potentiate the effect of ADH on renal tubules, probably by inhibiting c-AMP breakdown. c-AMP serves as an intracellular mediator of ADH and induces the ultimate functional change, the increase in water permeability of the tubular epithelium (Orloff and Handler, 1967; Dousa, 1973). Exogenous c-AMP added to the tissue mimics the hydroosmotic effect of ADH both in renal tubules and in amphibian bladders, such as the toad bladder (Orloff and Handler, 1962; Grantham and Burg, 1966).

Terminal events at the luminal membrane.

The mechanisms by which c-AMP, formed within the cell under the influence of vasopressin, produces the change in water permeability have not been fully elucidated. By current view the final event may be the insertion of water permeable patches into the apical (luminal) membrane (Muller and Kachadorian, 1984; Hays et al., 1987; Brown, 1989).

In amphibian bladder, Chevalier and co-workers (1974) and later others (Kachadorian et al., 1977) have demonstrated by freeze-fracture electronmicroscopy that an increased transepithelial flow

of water, induced by neurohypophyseal hormones (including vasopressin), is associated with the appearance of intramembranous particle (IMP) clusters (or aggregates) within the apical membranes of responsive cells. The same phenomenon was demonstrated in mammalian collecting ducts also (Harmanci et al., 1978). In both amphibian and mammalian epithelia, the number of particle aggregates per unit of area of apical membrane was proportional to the concentration of ADH (Kachadorian et al., 1977; Harmanci et al., 1978, 1980). Consistently, only a few particle aggregates were visualized in renal papillas of Brattleboro rats, known to lack ADH, until these animals were infused with the hormone (Brown and Orci, 1983). Furthermore, IMP clusters are absent from the apical membranes of collecting duct cells from mice with severe NDI (Brown et al., 1985). Based on the aforementioned observations, it is generally assumed that particle aggregates constitute or are closely associated with ADH-elicited water channels. Initially, these IMPs were attributed to the aggregation of intramembranous particles that were present under basal conditions. Recently, it was indicated that, at least in amphibian bladders, these particles originate from elongated cytoplasmic vesicles, termed aggregophores (Muller and Kachadorian, 1984). Upon stimulation with ADH, aggregophores fuse with the apical membrane and after fusion, a proportion of the aggregophore's component of particle aggregates is translated into the lipid bilayer of the apical membrane. Removal of ADH causes detachment of aggregophores from the apical membrane and a rapid decrease in tissue water permeability (Muller and Kachadorian, 1984). To gain more information about the molecular weight of vasopressin-induced proteins in aggregophores that subsequently appear on the cell surface, Harris and co-workers (1988) have used, in the toad bladder, a combination of lactoperoxidase-induced ionidation and horseradish peroxidase density shifting of endocytosed vesicles. The density-shift technique is a method for increasing the density of vesicles so that they can be more easily separated from other cell components by centrifugation. A number of candidate bands have been identified by gel electrophoresis and monoclonal antibodies are being prepared against specific bands.

Aggregophores have not been visualized in collecting duct cells of the mammalian kidney. The details of the ADH-elicited apical membrane events in the mammalian collecting duct are less clear. The current hypothesis on this proces was recently published by Brown (1989). In the collecting duct, the intramembranous particle (IMP) clusters, presumed to represent water channels, are located in pits coated by clathrin, a unique membrane protein, in the apical surface of principle cells. The principal cell is one of two major cell types in the collecting duct (the other one being the intercalated cell). Principal cells account for 60-70% of the cells in the cortical collecting duct and are characterized by extensive basilar membrane infoldings. Vasopressin induces a large increase of both IMP clusters and coated pits in collecting ducts of Brattleboro rats (Brown and Orci, 1983). Exocytotic insertion of the

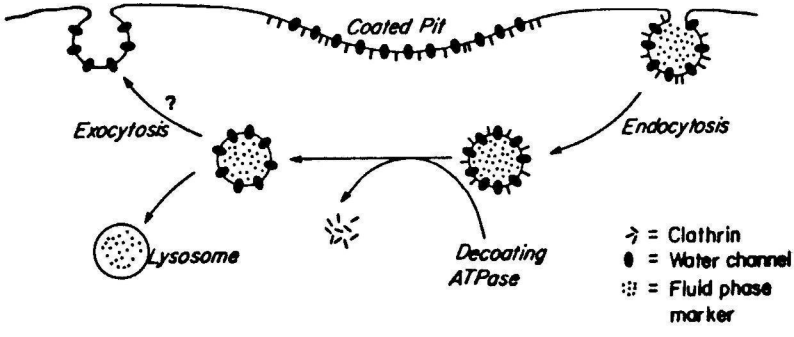

Fig. 1.7:
Diagram of the proposed pathway of water channel recycling in collecting duct principal cells. IMPs that are believed to represent water channels are concentrated in clathrin-coated pits at the cell surface and are endocytosed in coated vesicles. These vesicles are rapidly decoated, and while their fluid-phase content may eventually reach multivesicular lysosomes, water channels themselves may escape degradation and be recycled back to the apical membrane.
(Taken from Brown, D. - Membrane recycling and epithelial function. Am. J. Physiol. 256, 1989, F1-F12, with permission)

water channels into the apical membrane is responsible for increasing the permeability to water (Abramow et al., 1987). As long as vasopressin is present, these water channels are continuously recycled to and from the apical membranes via coated pits (Fig.7). Upon vasopressin withdrawal, the endocytotic pathway takes precedence, so that the coated patches, containing the water channels are rapidly removed from the apical membrane and reside on vesicles in the cytoplasm (Brown et al., 1988). Evidence for this latter event was given by Strange et al.(1988) by studying the endocytosis of horseradish peroxidase in perfused cortical collecting tubules. They demonstrated increased apical membrane endocytosis via coated pits to occur following removal of ADH from the peritubular bathing medium. They suggested that the rapid decline in the permeability of the cortical collecting duct, which takes place in response to ADH withdrawal, is mediated by retrieval of water permeable membrane via coated pits. Thus, sufficient evidence exists that clathrin-coated pits are involved in cluster internalization in the kidney. However, the mechanisms underlying this process at the molecular level are completely unknown at present.

Intermediate events triggered by c-AMP.

The intermediate steps between the generation of c-AMP and the final permeability changes in the apical membrane, described above, are not well defined.

Protein kinase

It is widely accepted that c-AMP activates a protein kinase, and that this activation is a prerequisite for its effect on water permeability (Jard and Bastide, 1970; Schlondorff and Franki, 1980; Edwards et al., 1980). Initially, it was believed that this c-AMP dependent protein kinase controls the phosphorylation of critical cellular proteins (Dousa and Valtin, 1976). This reaction could be reversed by phosphoprotein phosphatase-catalyzed dephosphorylation of the effector proteins. However, these putative effector phospho-

proteins have proven extremely difficult to identify. Reports of specific c-AMP dependent phosphorylation of apical cell membrane in bovine renal medulla (Schwartz et al., 1974) have not been confirmed. A 50,000 dalton protein, found on toad urinary bladders and initially linked to the cellular actions of ADH and c-AMP, appeared to be identical to the regulatory subunit of protein kinase itself (Malkinson et al., 1975). It is now known that this c-AMP dependent protein kinase (cA-PK) exists as two major isoenzymes (Lohmann and Walter, 1984), the cA-PK type I and type II, which differ in the structural and functional properties of their cAMP-binding subunit (R_I and R_{II}, respectively). Recently, Gapstur and co-workers (1988), utilizing the technique of photoaffinity labeling with ^{32}P-labelled 8-azido-adenosine 3'5'-cyclic monophosphate (N_3-8[^{32}P] cAMP) as a probe, provided direct evidence that cAMP binds to the regulatory subunits R_I and R_{II} of cAMP dependent protein kinase in medullary collecting duct and thick ascending limb of the rat kidney. Their analysis showed incorporation of N_3-8[^{32}P]cAMP into two bands (M_r=40,000 and M_r=55,000) that co-migrated with the cA-PK subunits R_I and R_{II}.

Cytoskeleton

Microtubuli and microfilaments might be involved in the hydroosmotic response to ADH and c-AMP (Kachadorian et al., 1979). Both organelles are important components of the cytoskeleton and are involved in the maintenance of cell shape and in the movement of cell components (Dedman et al., 1979). Several drugs that are known to disrupt microtubular systems, such as colchicine and vinblastine, inhibit the action of vasopressin and c-AMP on water permeability in the toad urinary bladder (Taylor et al., 1973). Cytochalasin, a microfilament inhibitor, has similar effects (Kachadorian et al., 1979). This suggests that the integrity of the cytoskeleton in epithelial cells is required in cellular action of ADH subsequent to c-AMP generation. However, the precise role of microtubules and microfilaments in the hydroosmotic response to vasopressin has yet to be delineated.

Calcium-calmodulin

Calcium has been suggested to modulate the antidiuretic action of ADH. Although there are some conflicting reports (Hardy, 1978; Dillingham et al., 1987; Taylor et al., 1987), the current view is that an increase in cellular calcium inhibits water flow in ADH-sensitive epithelia. Both a pre- and post c-AMP mechanism has been implicated. Cellular calcium concentrations modulate the activity of the adenylate cyclase. In medullary collecting duct cells of the rat, Teitelbaum and Berl (1986) demonstrated that lowering of cellular calcium by EGTA was associated with an increment in cAMP generation, while an elevation of cellular calcium by the calcium ionophore A23187, was consistently associated with an impairment in the response to vasopressin. On the other hand, a post c-AMP mechanism, most likely related to an effect of Ca^{2+} (in complex with calmodulin) on microtubular structures, has been proposed (Dillingham et al., 1987).

Atrial natriuretic factor

Atrial natriuretic factor (ANF) has been shown to counteract the antidiuretic effect of vasopressin (Dillingham and Anderson, 1986; Nonoguchi et al., 1988). However, the level of interaction between the two hormones remains to be elucidated. Both a site proximal (Dillingham and Anderson, 1986) and distal (Naray-Fejes-Tóth et al., 1988; Nonoguchi et al., 1988) to the generation of c-AMP have been suggested.

2. Modulation of solute transport.

In addition to the modulation of water transport in the collecting duct system, vasopressin increases urea transport in the terminal part of the mammalian inner medullary collecting duct (Morgan and Berliner, 1968; Sands et al., 1987). It has been demonstrated that the vasopressin-mediated increase in urea permeability in this nephron segment also results from activation of adenylate cyclase and generation of intracellular c-AMP (Star et al., 1988). This urea transport is important for the urinary concentrating mechanism

because it supplies nearly all of the urea that accumulates in the medullary interstitium (Sands and Knepper, 1987) (see 1.2.3: countercurrent multiplication).
In various non-human kidneys (rat; mouse; rabbit), vasopressin stimulates reabsorption of NaCl in the thick ascending limb of Henle's loop (Sasaki and Imai, 1980). Such a process could have an important role in the renal concentrating mechanism, by enhancing solute accumulation in the medullary interstitium. In these species the presence of a vasopressin-sensitive adenylate cyclase in the medullary portion of the thick ascending limb has been demonstrated (Imbert-Teboul et al., 1978; Morel, 1981). In humans, however, this nephron segment appeared to be completely unresponsive to ADH (Morel, 1981). Therefore, it seems unlikely that the transport of NaCl in the human thick ascending limb is modulated by ADH.

In summary, the antidiuretic effect of vasopressin can be attributed mainly to its influence on the water permeability of the collecting duct system. ADH-induced increase in urea permeability of the terminal inner medullary collecting duct and, in some species (but not in man), the increase in NaCl reabsorption abet the build-up of a cortico-papillary osmotic gradient and thus improve the concentrating ability of the kidney.

1.2.5 EXTRARENAL EFFECTS OF VASOPRESSIN

In addition to its effects on the kidney, vasopressin exerts a panoply of extrarenal functions, the most important of which will be discussed here.

1. Cardiovascular regulation
Vasopressin has a significant role in cardiovascular regulation. Reduction in blood volume and blood pressure are potent stimuli for vasopressin release (see 1.2.2). Vasopressin is a potent vasoconstrictor in vitro. As early as 1922, Krogh and Rehberg, in studies of the circulation in the web of the frog's foot, recognized the great vasoconstrictoring potency of an extract of the

posterior pituitary. They suggested that this gland might secrete a substance which circulates in the blood at a very low concentration and which contributes to the maintenance of the tonicity of the capillaries. The actions of ADH on the cardiovascular system are very complex. An excellent review on this subject was recently published by Share (1988). Vasopressin causes vasoconstriction in most vascular beds (Nakano, 1973), with the exception of the cerebral vessels (Lasoff and Altura, 1980). The receptors responsible for the action of vasopressin on vascular smooth muscle are classified as V_1 receptors (see 1.2.1).

In addition to its vasoconstrictoring effect, vasopressin acts on the hart in two important ways. It decreases both the heart rate and the cardiac output (Lee et al., 1988). These cardiac effects result from very small, physiologically relevant elevations in plasma vasopressin levels (Aylward et al., 1986; Ebert et al., 1986; Tipayamontri et al., 1987). The mechanisms by which these effects on the heart are achieved are in part understood. Baroreflex mechanisms appear to contribute to lowering cardiac output in conscious dogs, since the fall is greatly blunted following sinoaortic baroreceptor denervation (Montani et al., 1980). As a result of the reflexively mediated decrease in heart rate and cardiac output secondary to a rise in peripheral vascular resistance, blood pressure in the intact organism does not change until vasopressin concentrations reach the upper end of the physiological range (Montani et al., 1980). High vasopressin concentrations are seen in situations of severe volume insufficiency, such as dehydration, hemorrhage, adrenal insufficiency or septic shock. In these circumstances, vasopressin has an important role in maintaining blood pressure (Laycock et al., 1979; Andrews and Brenner, 1981). When blood volume is reduced, vasopressin acts to maintain blood pressure in conjunction with the sympathetic nervous system and the renin-angiotensin-aldosterone system (McNeill, 1983; Berecek et al., 1985). In addition, there is evidence for interactions between vasopressin and other systems in the regulation of cardiac function: Vasopressin stimulates the synthesis of prostaglandin I_2 (PGI_2), a potent vasodilatator, from vascular smooth

muscle (Hassid and Williams, 1983), and PGI_2 in turn modulates the vasoconstrictoring action of vasopressin (Glänzer et al., 1982).
Vasopressin causes relaxations of isolated basilar arteries of the dog, an effect that is dependent on the presence of endothelium (Katusic et al., 1984). This is due to a vasopressin induced release of a humoral factor termed endothelium derived relaxing factor (EDRF), which relaxes the underlying vascular smooth muscle. However, in the femoral artery, endothelial cells do not modulate the contractile response to vasopressin. The latter finding confirms that vasopressin acts as a potent endothelium-independent constrictor on peripheral blood vessels. The differential effects of vasopressin on cerebral and peripheral arteries support the interpretation that increased levels of circulating vasopressin, for instance during hemorrhage or septic shock, favor the redistribution of blood from the periphery to the cerebral circulation and help to maintain cerebral blood flow (Katusic et al., 1984).
Interactions between vasopressin and atrial natriuretic factor (ANF) have been described. In addition to its natriuretic and diuretic properties, ANF has a potent vasodilatory effect (DeBold, 1985), opposite to the vasoconstriction induced by vasopressin. Based on experiments with rat aortic smooth muscle cells, Nambi and co-workers (1986) suggested that the vasoconstrictor activity of vasopressin might partly involve inhibition of ANF-receptor-mediated vascular relaxation, through inhibition of accumulation of the second messenger, cyclic guanosine monophosphate (c-GMP). However, the exact molecular mechanism(s) of interaction between vasopressin and ANF remains to be elucidated.
Finally, the gonadal steroid hormones can substantially affect the cardiovascular actions of vasopressin. Thus, the pressor responsiveness is much greater in male than in randomly cycling female rats (Crofton et al., 1986). The mechanisms responsible for the differences in pressor responsiveness between males and females are uncertain, but could involve actions of the gonadal steroids on the central nervous system and the peripheral vasculature.

2. Platelet aggregation

In platelets vasopressin has been shown to induce shape change, aggregation and release reaction (Haslam and Rosson, 1972). The platelet vasopressin receptor appeared to be of the V_1 subtype, since vasopressin induced aggregation was potently inhibited by V_1 antagonists, whereas a selective V_2 agonist failed to induce aggregation of human platelets (Thomas et al., 1983; DiTullio et al., 1985). In the circulation, however, vasopressin concentrations are in the pM range (Share, 1988), which means that physiologically only a fraction of platelet vasopressin receptors are occupied and/or activated. Therefore, it remains to be established whether or not this vasopressin receptor has any functional significance in terms of platelet pathophysiology.

3. Glycogenolysis

Vasopressin elicits a glycogenolytic and gluconeogenetic response in rat hepatocytes (Hems and Whitton, 1973; Tolbert et al., 1980). This effect is mediated also by vasopressin receptors of the V_1 subtype. The rat liver V_1 receptor has recently been purified and characterized (Fishman et al., 1987). This receptor is a glycoprotein and has a molecular weight of approximately 68,000 under reducing conditions and 58,000 under non-reducing conditions.

4. Miscellaneous

Vasopressin increases corticotropin release by the adenohypophysis (Gillies et al., 1978), has mitogenic effects on several cell types (Whitfield et al., 1970; Hunt et al., 1977; Rozengurt et al., 1979), and increases the firing rate of various neuronal groups in the brain (Mühlethaler et al., 1982).

In addition, vasopressin has several effects on animal behaviour. A number of experiments in rats have shown that vasopressin maintains learned avoidance behaviour, reverses amnesia and supports other aspects of memory function (De Wied 1969). Vasopressin and vasopressin analogues appear to be involved in various aspects of memory consolidation both in experimental animals and humans. A review on this last subject is given by De Wied (1976).

Vasopressin and its V_2 agonist 1-desamino-8-D-arginine vasopressin (DDAVP) are known to produce an increase in blood clotting factor VIII-, von Willebrand factor (vWF)- and tissue-type plasminogen activator levels in man (Manucci et al., 1975). It has been suggested that these effects are mediated through stimulation of extrarenal V_2 receptors. This hypothesis was supported by the observation that FVIII, vWF (Kobrinski et al., 1985; Bichet et al., 1988) and plasminogen activator (Knoers et al., 1990) were not stimulated by DDAVP in patients with nephrogenic diabetes insipidus. A detailed discussion on these extrarenal effects and absence of these effects in NDI patients will follow later (Chapter 5 and 6).

1.2.6 ANIMAL MODEL FOR N.D.I.

A mutant mice strain, DI severe, has been described (Naik and Valtin, 1969) which is a phenocopy of human NDI and is characterized by an inherited, vasopressin-resistant defect of urine concentration. Biochemical studies in these mice have helped to delineate the specific defects for the ADH resistance in this animal model. Dousa and Valtin (1974) measured the activity of adenylate cyclase in cell-free membrane preparations from renal medulla in the absence of hormone (basal activity), in the presence of 10^{-6} M vasopressin for maximal stimulation, and after nonspecific stimulation with fluoride in DI severe mice. The activity of adenylate cyclase, when stimulated maximally by saturating doses of vasopressin, was found to be significantly lower in DI severe mice than in control animals. The basal and fluoride-stimulated activities of renal medullary adenylate cyclase in these mice did not significantly differ from those in normal mice, indicating that there is no major deficiency in the catalytic capacity of adenylate cyclase itself. Furthermore, the adenylate cyclase of DI severe mice could be stimulated by vasopressin, and the dose required to elicit one-half of the maximal stimulation was not significantly different from the dose in controls. These findings suggest that

the receptor has normal affinity for vasopressin. In addition, c-AMP phosphodiesterase and c-AMP dependent protein kinase activities were not different from those of normal mice.

More recently, Jackson et al (1980b), in experiments carried out in microdissected tubules, found the functional unresponsiveness of DI severe mice to vasopressin is mainly the result of the inability of the collecting tubules to increase intracellular c-AMP levels in response to vasopressin. This inability was assumed to result partly from lower stimulation of adenylate cyclase in the medullary collecting tubule, but mainly from abnormally high c-AMP phosphodiesterase activity in the medullary collecting tubule, since inhibition of c-AMP phosphodiesterase with 1-methyl-3-isobutyl xanthine (MIX) caused a significant increase of vasopressin-induced c-AMP levels. In addition, ADH-stimulated adenylate cyclase was found to be significantly reduced in the medullary thick ascending limb of Henle's loop. Based on these observations, they suggested that the decreased adenylate cyclase, demonstrated in the studies of Dousa and Valtin (1974), was due to decreased activity of this enzyme in the thick ascending limb rather than in the collecting ducts. The decrease in adenylate cyclase activity in the thick ascending limb of hereditary DI severe mice might represent the biochemical basis of a second abnormality in the concentrating ability of these animals (i.e., a defect in gradient formation). However, since an ADH-sensitive adenylate cyclase has not been detected in the human thick ascending limb (Morel, 1981), the relevance of these findings for hereditary NDI in humans is unknown.

Recently, Kusano et al. (1986) reported that deficient ATP, the substrate for c-AMP generation by adenylate cyclase, is not a limiting factor accounting for defective ADH-stimulated c-AMP generation in medullary collecting tubules of DI severe mice. This is consistent with the hypothesis that the defective cellular activity of vasopressin in these mice is due to elevated levels of c-AMP phosphodiesterase, resulting in increased c-AMP degradation. Brown et al. (1985) argued that the defect could be localized at other sites of the metabolic cascade as well. They demonstrated

lack of intramembranous particle cluster aggregation in collecting ducts of mice with hereditary DI.
Very recently, Braun and Stallone (1989) reported a second good animal model that can be exploited to gain better understanding of the mechanism(s) underlying NDI. This model is a strain of White Leghorn domestic fowl that appeared to possess all the characteristics of hereditary NDI. The defect in these birds is inherited as an autosomal recessive trait.

1.2.7 POSSIBLE MECHANSIMS UNDERLYING THE UNRESPONSIVENESS TO ANTI-DIURETIC HORMONE IN NDI

At present, the pathogenesis of the concentrating defect in patients with NDI is totally unknown. In contrast to patients with central diabetes insipidus, no defects in ADH synthesis or release have been found in any of the NDI patients (Williams and Henry, 1947; Luder and Burnett, 1954; Cannon, 1955; Holliday et al., 1963). In addition, anti-ADH-antibodies are absent from the plasma of these patients (Forssman, 1945; Robertson and Scheidler, 1981). Following Williams and Henry (1947), disturbances in the generation and/or maintenance of an osmotic gradient (see 1.2.3) can not be responsible for the defective concentrating ability in 'true' NDI. Consequently, familial NDI appears to be exclusively due to renal resistance to the action of ADH. Theoretically, in view of the aforementioned physiological aspects of ADH action, the defect in NDI could be located at any of the steps from vasopressin-binding to the final effect of the hormone on the luminal membrane. Initially, attempts were made to determine whether the primary abnormality is located proximal or distal to c-AMP generation, utilizing the urinary excretion of c-AMP after administration of vasopressin as an indicator of the renal action of the hormone (see also 1.1.3). Urinary levels of c-AMP in NDI patients did not increase in response to ADH in some reports (Bell et al., 1974; Usberti et al., 1980). On the other hand, a significant increase in c-AMP excretion after vasopressin has been reported by others

(Ohzeki et al., 1984). Based on these observations, Ohzeki (1985) divided NDI into two types with regard to the intrarenal site of ADH resistance. One type was considered to have a defect in the production of c-AMP in renal tubular cells, and the other in the reception of the c-AMP signal. However, it has been demonstrated that urinary c-AMP excretion does not reflect intracellular c-AMP levels accurately (Bia et al., 1979). Proesmans et al. (1975) utilized the administration of exogenous c-AMP to define the existence of defects distal to c-AMP generation. However, since the intravenous or intramuscular administration of c-AMP is followed by rapid inactivation of this compound by extrarenal tissues, a lack of response to exogenous c-AMP need not imply the presence of a distal defect. Thus, in spite of intensive study, direct evidence for a defect either proximal or distal to c-AMP generation in patients with NDI is lacking.

Defects proximal to the formation of c-AMP would include:

(1) Defective vasopressin binding due to absence or abnormality of the renal V_2 receptor.
(2) Decreased adenylate cyclase activity due either to defective activation and/or functioning of the guanine nucleotide binding proteins involved in adenylate cyclase activity, or due to a defect in the enzyme per se.
(3) Increased c-AMP phosphodiesterase levels, which would induce abnormally high degradation of c-AMP to AMP.

Until now, it has not been possible to directly measure vasopressin-binding, adenylate cyclase activity and its stimulation with vasopressin in human kidney specimens of NDI patients. An abnormally active c-AMP phosphodiesterase has been reported in DI severe mice (Jackson et al., 1980b). However, it should be recalled that, although the hereditary disease in mice has functional features similar to those of the hereditary disease in man, it is not certain that the defect in cellular action of ADH in human NDI is identical or analogous to that in the experimental disease.

Defects distal to the generation of c-AMP could include:

(1) Inhibition or defect of c-AMP dependent protein kinase .
(2) Disruption of microtubules and/or microfilaments.
(3) Abnormalities in intramembranous particle aggregation.
(4) Alterations in calcium homeostasis.
(5) Alterations in cellular prostaglandin metabolism.

Of all these potential defects distal to c-AMP generation only the abnormality in intramembranous particle aggregation has been observed in the mouse NDI model (Brown et al., 1985).
Whether lack of intramembranous particle aggregation is involved in the concentrating defect in NDI patients remains to be elucidated. Since disruptions of the cytoskeleton and alterations in calcium homeostasis are expected to involve not only the kidney they can be readily excluded as possible candidates for the defect underlying NDI. The presumed role of prostaglandins in the pathogenesis of NDI has been discussed in detail in 1.1.5. For further discussion of the possible defect(s) in NDI, we refer to chapter 8.

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1.4 AIM OF THE STUDY.

Reliable detection of women heterozygous for X-linked Nephrogenic Diabetes Insipidus is of considerable importance, because this offers the possibility of treating affected males very early in life. Early diagnosis of affected males is essential because, without treatment, severe brain dehydration may lead to mental retardation. Female NDI carriers are clinically asymptomatic but may demonstrate a defect in urinary concentrating ability. However, normal concentrating ability does not rule out the possibility that the female is a carrier of the disease.

The fact that the NDI gene lies on the X chromosome offers possibilities for identification of carriers and, possibly, prenatal diagnosis, with use of DNA recombinant technology. Therefore, we employed DNA linkage analysis in 11 Dutch NDI families (Chapter 2, 3, and 4).

There is evidence that the renal insensitivity to vasopressin in NDI is due to a defect in the renal V_2 receptor. It has been postulated that the V_2 receptor defect is not confined to the distal nephron, but is equally expressed in extrarenal tissues. In order to test this hypothesis and with the aim of getting more insight into the pathogenesis of NDI, we studied the hemodynamic, coagulation, and fibrinolytic responses to the synthetic V_2 analogue 1-desamino-8-D-arginine vasopressin (DDAVP), effects assumed to be mediated through extrarenal V_2 receptor activation, in NDI patients and compared these responses to those found in normal controls (chapter 5 and 6). In addition, the fibrinolytic responses to DDAVP were examined in female NDI carriers in order to find out whether the reaction to DDAVP infusion could be of value in discriminating carriers from non-carriers (Chapter 5).

Since the responses to DDAVP in one of our patients appeared to be completely different from those found in the other patients, we assumed that this patient must suffer from a variant type of NDI. His case is presented in chapter 6.

The most effective treatment of NDI at the moment, indomethacin

combined with hydrochlorothiazide, has many disadvantages, such as hypokalemia due to prolonged use of hydrochlorothiazide and several other side effects inherent to long-term treatment with indomethacin. Based on a previous study suggesting the possible efficacy of the combination amiloride-hydrochlorothiazide in the treatment of NDI, we compared the effects of this alternative combination to those of the indomethacin-hydrochlorothiazide regimen (chapter 7).

CHAPTER 2

LINKAGE OF X-LINKED NEPHROGENIC DIABETES INSIPIDUS WITH DXS52, A POLYMORPHIC DNA MARKER

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LINKAGE OF X-LINKED NEPHROGENIC DIABETES INSIPIDUS WITH DXS52, A POLYMORPHIC DNA MARKER

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2.1. ABSTRACT

In five families with X-linked nephrogenic diabetes insipidus (NDI), linkage studies with the DNA marker DXS52, defined by probe St14, have shown no recombination with a maximum combined lod score score of 6.40. These results assign the NDI gene to the subtelomeric region of the X chromosome long arm. This finding should facilitate identification of carriers and should also be helpful in finding the NDI gene itself.

2.2. INTRODUCTION

Nephrogenic diabetes insipidus (NDI) is a rare X-linked disorder, characterized by insensitivity of the renal tubule to antidiuretic hormone. Affected males usually present soon after birth with polyuria, severe dehydration, fever, and failure to thrive. Without treatment some patients die and of those surviving, many are mentally retarded. The biochemical defect underlying NDI is still unknown.

Female carriers are clinically asymptomatic but may demonstrate a defect in urinary concentrating ability [1]. This allows detection of a certain proportion of carriers. However, normal renal concentrating ability does not rule out the possibility that the female is a carrier of the disease [2].

The regional assignment of the NDI gene on the human X chromosome is not yet known, although a previous study has excluded genetic linkage with the Xg blood group, which is located in the Xp22.3-Xpter region [3]. We performed linkage studies with various X-linked restriction fragment length polymorphisms (RFLPs), i.e. genetic markers that can be detected by restriction endonuclease digestion, electrophoretic separation of DNA fragments and hybridization with cloned DNA sequences, so-called probes [4].

Here we report on the results of linkage studies in five families with NDI employing the DXS52 marker, which is defined by the St14 probe [5].

Subjects

Included in the study were five families with NDI from the Netherlands. Pedigrees are shown in Fig. 1.

In affected males, diagnosis was confirmed by the lack of increase of urine osmolality after administration of the synthetic vasopressin analogue, 1-desamino-8-D-arginine-vasopressin (DDAVP). Maximal urine osmolalities that could be achieved after DDAVP infusion were 51-198 mosmol/kg (mean 104 mosmol/kg).

Twelve affected males and 58 unaffected family members were available for RFLP analysis.

DNA analysis

Blood samples (40 ml) from affected and unaffected family members were collected in EDTA and stored at -20°C until use. Total human DNA was isolated by the following method: 20 ml of blood was added to 200 ml lysis buffer (0.32 M sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl; pH 7.5) and centrifuged at 2,800 g for 10 min at 4°C. The supernatant was carefully aspirated, taken up in 50 ml of the same lysis buffer and the centrifugation was repeated. The pellet of white cell nuclei was resuspended in 9 ml of 75 mM NaCl, 24 mM EDTA (pH 8). To this was added 450 µl 10% sodium dodecyl sulfate (SDS) and 500 µl of 3 mg/ml proteinase K (Boehringer, Mannheim) in 0.2 M Tris-HCl (pH 8.0), 0.2 M EDTA, 0.5% sodium dodecyl sulfate and the mixture was incubated overnight at 50°C. To remove proteins, the digest was extracted with an equal volume of phenol, followed by extraction with phenol/chloroform (1:1) and subsequently with pure chloroform. The DNA was precipitated with 0.75 volume of isopropanol and 0.01 volume of 100 µl 5 M NaCl and washed twice with 70% ethanol. The DNA samples were resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and stored at 4°C. The DNA (10 µg) was digested overnight at 50°C with the restriction enzyme TaqI (Boehringer, Mannheim), separated on 0.9% agarose gel, denatured in 0.25 N HCl and transferred to Gene screen plus filters (NEN research products, Boston) in 0.5 M NaOH, 1.5 M NaCl by the Southern Blot method [6].

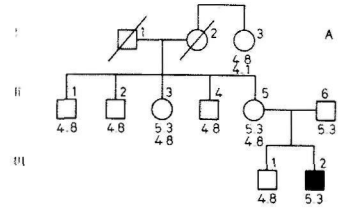
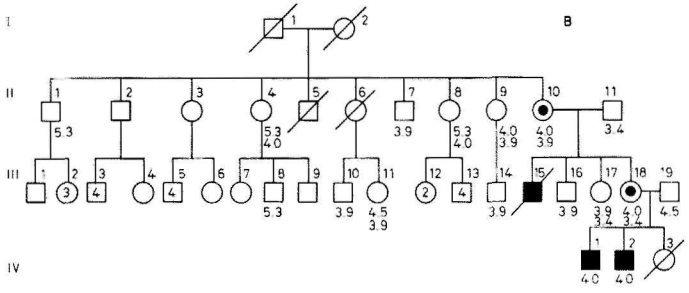
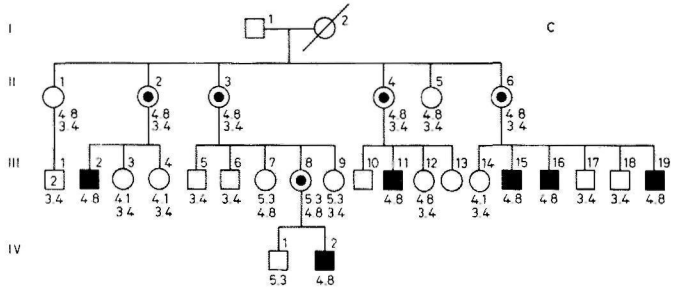
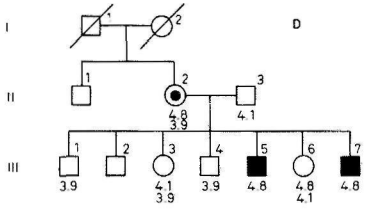
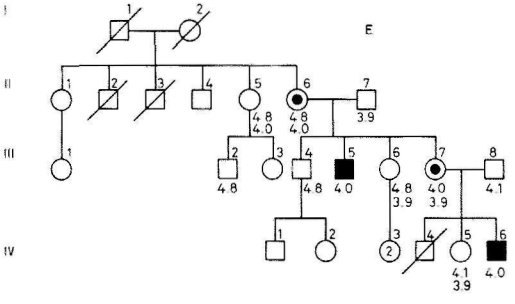


Fig 2.1 : Pedigrees of five families with X-linked NDI A-E. Closed squares = affected males; circles with central dot = obligatory carriers. Numbers refer to the length of allelic restriction fragments detected with the St14 probe. In family C, NDI co-segregates with the 4.8-kb allele. Absence of this allele in possible carriers may indicate that these females do not carry the NDI gene.









After transfer, filters were rinsed in 50 mM phosphate buffer and irradiated with ultraviolet light for 2 min. Filters were prehybridized for 4-6 h at 65°C in 0.5 M sodium phosphate buffer (pH 7.3), containing 7 % SDS, 1 mM EDTA, and 500 μg sonicated herring sperm DNA. The St14 probe (kindly provided by J.L. Mandel, Strasbourg) (200-400 ng) was radiolabeled with 5 μl ^{32}P-dCTP to a specific activity of 2.5 x 10^8 cpm/μg using the primer elongation method [7]. The radiolabeled probe was denatured by heating to 100°C. Subsequently it was hybridized overnight at 65°C to DNA bound to the filters in 10 ml 0.5 M sodium phosphate buffer (pH 7.3), containing 7 % SDS, 1 mM EDTA and 250 μg sonicated herring sperm DNA. After overnight hybridization the filters were rinsed 3 times for 5 min and once for 30 min, respectively, in 40 mM sodium phosphate buffer (pH 7.3) and 1 % SDS at 65°C. The filters were then exposed to a Kodak X-Omat S film with two intensifying screens for 1-3 days at -70°C.

Linkage analysis

Linkage analysis was carried out using the LINKAGE program [8] on an IBM compatible personal computer. This program uses the method of maximum likelihood to calculate lod scores (log of odds of linkage versus independent assortment) [9] at selected recombination fractions for each pedigree and also permits summing lod scores at each value for individual pedigrees. The frequency of the mutant NDI allele in females was taken as 1 in 10^5 and a mutation rate of 5×10^{-6} was assumed. For linkage analysis, biochemical abnormalities in possible female carriers were not taken into account.

2.4. RESULTS

When human genomic DNA is digested with the restriction enzyme TaqI, the St14 probe reveals a complex polymorphic banding pattern with up to 10 allelic fragments ranging from 6.6 to 3.4 kb and several constant bands [5]. In all our five families the obligate carriers of NDI were informative for the St14 marker, that is, hy-

bridization with the St14 probe revealed restriction fragments of differing size on their X chromosomes, which permitted distinction between the X chromosome bearing the NDI gene and the normal chromosome. Affection status as well as genotypes of all individuals tested are shown in Fig.1. In each family, specific (but mostly different) St14 alleles cosegregate with the disease. For example, in family E, the TaqI 4.0 kb allele was always associated with the NDI gene through three generations. This disease-specific allele was not found in the unaffected male offspring. The lod score for this family was 1.03 at a recombination fraction of 0.00 (Table 1).

Table 2.1 Lod scores for linkage relationship between NDI and DXS52 in five families at different values of recombination fraction

	Recombination fraction , θ							$\hat{Z}^a$	$\hat{\theta}^b$
	0.00	0.01	0.05	0.10	0.20	0.30	0.40		
Family A	0.44	0.43	0.40	0.35	0.26	0.16	0.08	0.44	0.00
Family B	1.26	1.24	1.14	1.02	0.76	0.50	0.24	1.26	0.00
Family C	2.77	2.75	2.65	2.46	1.97	1.35	0.61	2.77	0.00
Family D	0.90	0.89	0.81	0.72	0.52	0.30	0.09	0.90	0.00
Family E	1.03	1.01	0.93	0.82	0.60	0.36	0.14	1.03	0.00
Totals	6.40	6.32	5.93	5.37	4.11	2.67	1.16	6.40	0.00

a Maximum likelihood estimation of the lod score.
b Recombination fraction at $\hat{Z}$.

No crossovers were observed between NDI and St14 in any of the five families. The combined maximum lod score was 6.40 at a recombina-

tion of 0 % (confidence limits: 0-10 %): that is, the odds were $10^{6.4}$:1 in favor of linkage.

2.5. DISCUSSION

The application of RFLPs as genetic markers has led to a detailed map of the human X chromosome [10]. This enables localization of almost any X-linked disorder if informative families are available for linkage studies. Localization of the defective gene and identification of closely linked polymorphic markers are useful for carrier detection and prenatal diagnosis especially if the biochemical defect underlying the disease is not known.

So far, linkage analysis with DNA probes has been succesful in determining the chromosomal localization of a great number of disorders, including Duchenne muscular dystrophy [11], Norrie's disease [12], X-linked retinitis pigmentosa [13] and adrenoleukodystrophy (ALD) [14].

As to NDI, a previous study has excluded linkage of the NDI locus with the Xg blood group, located in the Xp22.3-pter region [3]. The present study demonstrates linkage between the gene responsible for NDI and the DXS52 locus, defined by probe St14, which maps at Xq28 [5]. The calculated maximum combined lod score of 6.40 at a recombination fraction of 0 % is highly significant. These results assign the NDI locus to the subtelomeric region of the X chromosome long arm in the vicinity of factor VIII, ALD, glucose-6-phosphate-dehydrogenase and the colour blindness genes (Fig.2). Whether the gene is distal or proximal to the DXS52 locus will be determined by further studies.

Until now only a certain proportion of NDI carriers could be identified by clinical testing owing to the fact that the heterozygote females do not always manifest a defect in renal concentrating ability [15]. The regional assignment of the gene responsible for NDI provides a closely linked marker, which may be of use for identification of carriers. This is exemplified for one family in Fig. 1. In addition, mapping of the NDI locus may pave the way towards

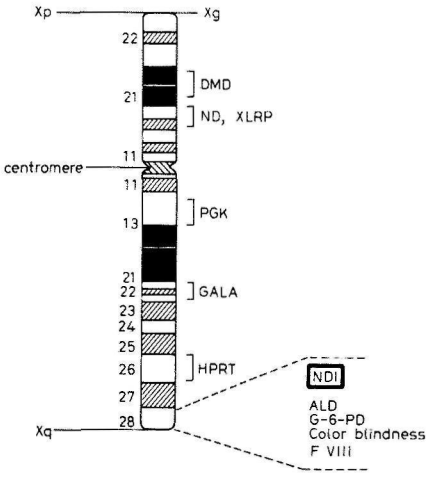


Fig. 2.2: Map of the X chromosome showing the position of various gene loci. The proposed site of the gene for X-linked NDI is indicated. PGK = phosphoglycerate kinase; GALA = α-galactosidase; HPRT = hypoxanthineguanine phosphoribosyltranseferase; DMD = Duchenne muscular dystropy; ND = Norrie's disease; XLRP = X-linked retinitis pigmentosa; G-6-PD = glucose-6-phosphate dehydrogenase; FVIII = factor VIII.

isolating the gene itself.

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NEPHROGENIC DIABETES INSIPIDUS: CLOSE LINKAGE WITH MARKERS FROM THE DISTAL LONG ARM OF THE HUMAN X CHROMOSOME

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NEPHROGENIC DIABETES INSIPIDUS: CLOSE LINKAGE WITH MARKERS FROM THE DISTAL LONG ARM OF THE HUMAN X CHROMOSOME.

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3.1 ABSTRACT

Ten families with Nephrogenic Diabetes Insipidus (NDI) have been analysed for restriction fragment length polymorphisms (RFLPs). A search for linkage was performed using various chromosome-specific single-copy DNA probes of known regional assignment to the human X-chromosome. Close linkage was found between the disease locus and the markers DXS52, DXS15, DXS134 and the F8 gene. This result assigns the NDI gene to the subtelomeric region of the long arm of the X chromosome. The regional localization of the gene by the identification of closely linked markers should have repercussions for genetic counselling and prevention in NDI families.

3.2 INTRODUCTION

Nephrogenic Diabetes Insipidus (NDI) is a rare inherited disorder, which was first described by Waring et al. (1945). Pedigree analyses indicate that the disease is transmitted by a single X-linked recessive gene (Williams and Henry, 1947; Bode and Crawford, 1969), although other modes of inheritance have been suggested (Robinson and Kaplan, 1960).

NDI is characterized by renal resistance to the antidiuretic action of vasopressin. As a consequence, the kidney loses its ability to concentrate urine. The disorder becomes manifest early in infancy, with polyuria, periods of life-threatening dehydration, fever, anorexia, vomiting and failure to thrive. Without treatment, mental retardation may develop due to brain dehydration. The gene involved has not yet been identified and the molecular pathogenesis of NDI is unknown. However, it has been suggested that the defect underlying vasopressin resistance is located beyond the intracellular formation of cyclic AMP (Proesmans et al., 1975). Females are clinically asymptomatic, but in 60 % of them a relative inability to concentrate urine can be demonstrated (Carter and Simpkiss, 1956; Schoen, 1960).

The subregional localisation of the NDI gene on the human X-chro-

mosome is unknown. Close linkage to the Xg-locus has been excluded (Bode and Miettinen, 1970). Recently, we have obtained evidence for linkage between the NDI gene and marker DXS52 (St14), which is located at Xq28 (Knoers et al., 1987, 1988; see also Davies et al., 1987). In order to extend these preliminary observations and to search for useful diagnostic markers in the vicinity of the disease gene, linkage studies were performed in ten Dutch NDI families, employing seven DNA markers from the long arm of the X-chromosome.

In the present report our linkage data, which enable precise localization of the NDI gene on the human X-chromosome, are summarized. In addition, our results provide compelling evidence that NDI is due to a single X-linked recessive gene defect.

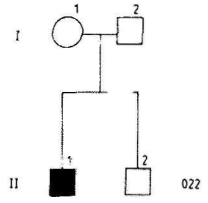
3.3 MATERIALS AND METHODS

Subjects

Ten NDI families from the Netherlands were included in the study. Pedigrees are presented in Fig.1. Nineteen patients and 95 family members were available for testing. All patients were males, four of them being sporadic cases, whereas in six families, transmission was compatible with X-linkage. Diagnosis of NDI was based on clinical symptoms and the lack of increase of urine osmolality after administration of the synthetic vasopressin analogue DDAVP (1-desamino-8-D-arginine vasopressin). Maximal urine osmolalities that could be achieved after DDAVP-infusion were 51-198 mosmol/kg (normal > 805 mosmol/kg).

DNA analysis

Venous blood samples (40 ml) from affected and unaffected family members were collected in EDTA and stored at -20^{o} C until use. DNA isolation was done as previously described (Knoers et al., 1988) : 20 ml of blood was added to 200 ml sucrose lysis buffer (0.32 M sucrose, 5 mM MgCl2, 10 mM Tris-HCl; pH 7.5) and centrifuged for 10 minutes at 4^{o}C and 2,800 g. The supernatant was aspirated and taken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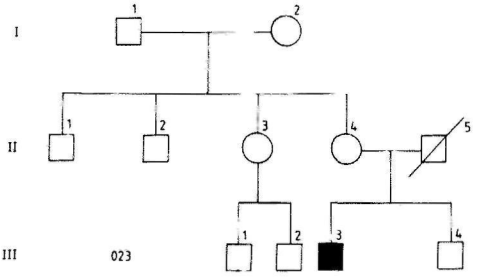
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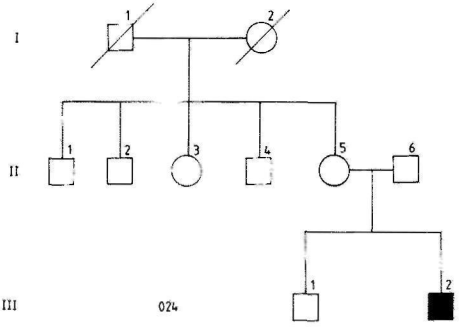
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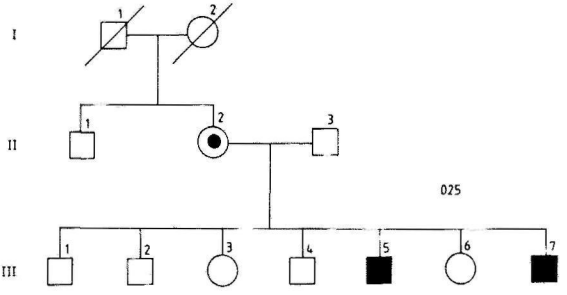
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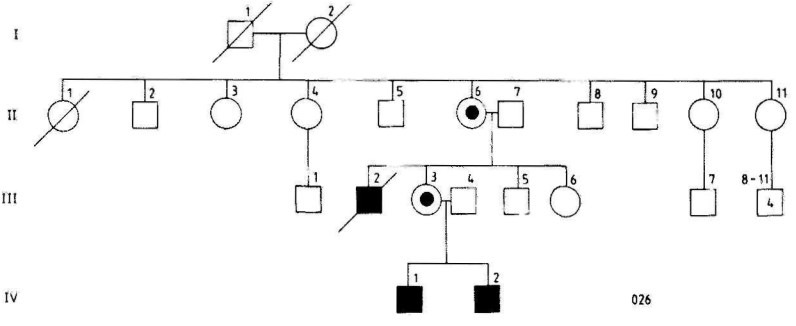
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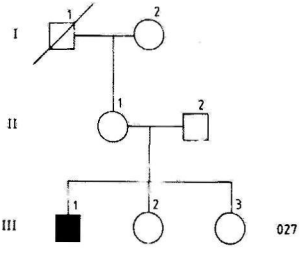
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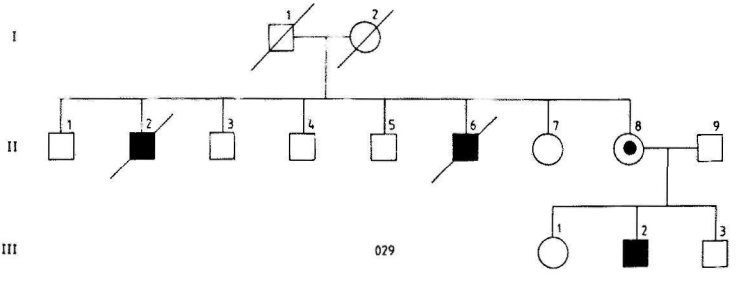
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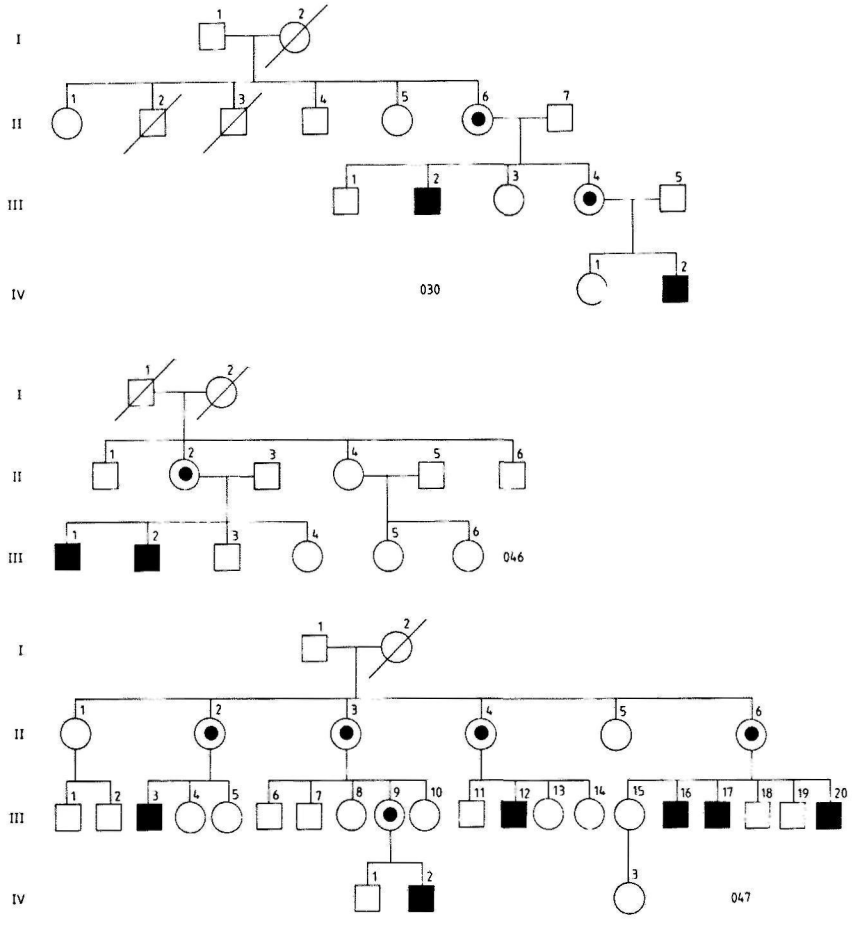

Fig. 3.1: Pedigrees of ten families with nephrogenic diabetes insipidus (NDI). ■ Affected males, ◉ obligatory carriers.

up in 50 ml lysis buffer. Then the centrifugation was repeated. The pellet of white cell nuclei was resuspended in 9 ml of 75 mM NaCl, 24 mM EDTA (pH 8.0). To this suspension 450 µl 10 % sodium dodecyl

sulfate (SDS) and 500 µl 3 mg/ml proteinase K (Boehringer, Mannheim) in 0.2 M Tris-HCl (pH 8.0), 0.2 M EDTA, 0.5 % SDS was added and the mixture was incubated at 50°C for 15-18 hours. The digestion mixture was extracted with an equal volume of phenol, followed by extraction with phenol:chloroform (1:1) and finally with pure chloroform. DNA was precipitated with 0.75 volume of isopropanol and 0.01 volume of 5 M NaCl, washed twice with ethanol, dissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and stored at 4°C. DNA samples were digested to completion using the appropriate restriction enzyme under conditions recommended by the suppliers. The resulting fragments were separated according to size by agarose gel electrophoresis (0.9 to 1.5 % agarose gel) and transferred to Gene Screen plus filters (NEN research products, Boston) by the Southern blot method (Southern, 1975). After the blotting, filters were rinsed in 50 mM phosphate buffer and irradiated with ultraviolet light for 1-2 minutes. DNA probes (200-400 ng) were radiolabelled with 50 µCi ^{32}P-dCTP to a specific activity of 2.5×10^8 cpm/µg using the primer elongation method (Feinberg and Vogelstein, 1983). Gene screen plus membranes were prehybridized for 4-6 hours at 65°C in 10 ml 0.5 M sodium phosphate buffer (pH 7.3), containing 7 % SDS, 1 mM EDTA with addition of 500 µg sonicated herring sperm DNA. Hybridization was performed overnight at 65°C in 10 ml prehybridization solution to which 250 µg sonicated herring sperm DNA and denatured probe had been added. The filters were washed three times for 5 minutes and once for 30 minutes, respectively, in 40 mM sodium phosphate buffer (pH 7.3) and 1 % SDS at 65°C.
Autoradiography was carried out at - 70°C using Kodak X-Omat S X-ray films for 1-3 days with intensifying screens.

Probes

Ten X-chromosome specific cloned DNA probes corresponding to the loci DXYS1, F9, DXS105, DXS15, DXS52, DXS134 and F8 were used in the linkage analysis. As shown in Table 1 each probe detects at least two allelic DNA fragments. For probe cpX.67 an MspI polymorphism has been described, which consists of two allelic bands of 3.7 and 3.4 kb (Hofker et al., 1987). In our family studies the

respective MspI fragments were slightly different, i.e. 4.1 and 3.2 kb.

Table 3.1 X-chomosome markers used in this study

Locus	Probe	Restriction enzyme	Allele sizes (kb)	Allele frequency	Genomic location	Reference
DXYS1	pDP34	*Taq*I	11 8 10 6	0 56 0 44	Xq13-q21	Page et al (1982)
F9	pVIII	*Taq*I	1 8 1 3	0 71 0 29	Xq26-q27	Connor et al (1986)
	pXIII	*Dde*I	1 75 1 70	0 24 0 76		Winship et al (1984)
	pVI 3	*Bam*HI/ *Eco*RI	8 0 6 3	0 94 0 06		Hay et al (1986)
DXS105	cX55 7	*Taq*I	5 3 3 5	0 16 0 84	Xq27-q28	Hofker et al (1987)
DXS15	DX13	*Bgl*II	5 8 2 8	0 45 0 55	Xq28	Drayna et al (1984)
DXS52	St14	*Taq*I	complex		Xq28	Oberlé et al (1985)
F8	p114 12	*Bcl*I	1 2 0 9	0 37 0 63	Xq28	Gitschier et al (1985)
	p482 6	*Kpn*I/ *Xba*I	6 2 1 4	0 41 0 59		Wion et al (1986)
DXS134	cpX 67	*Msp*I	3 7 3 4	0 70 0 30	Xq28	Hofker et al (1987)

Linkage analysis

Two-point linkage analysis was performed with the LINKAGE program (Lathrop and Lalouel, 1984) and lod scores were calculated for various values of the recombination fraction. For the mutant NDI allele a gene frequency of 1 in 10^5 and a mutation rate of 5×10^{-6} were assumed (Haldane, 1935).

Table 3.2 Relevant phenotypes of individuals studied (expressed in kilobase size of DNA fragments).nt, not tested

Kindred	Individual	Genotypes					
		DXYS1	F9	DXS15	DXS52	F8	DXS134
022	I 1	11 8/10 6	1 8/1 3[a]	5 8/2 8	5 3/4 1	1 2/0 9[d]	–
	II 1	10 6	1 8	5 8	4 1	0 9	–
	II-2	11 8	1 8	2 8	5 3	1 2	–
023	I-1	10 6	1 3[a]	nt	3 4	1 2[d]	4 1
	I-2	11 8/11 8	1 8/1 8	–	5 3/3 9	1 2/0 9	3 2/3 2
	II 1	nt	nt	–	5 3	nt	3 2
	II-2	11 8	1 8	–	3 9	0 9	3 2
	II-3	11 8/10 6	1 8/1 3	–	3 9/3 4	1 2/0 9	4 1/3 2
	II 4	11 8/10 6	1 8/1 3	–	5 3/3 4	1 2/0 9	4 1/3 2
	III-1	nt	1 8	–	3 9	nt	3 2
	III 2	nt	1 3	–	3 4	nt	4 1
	III-3	10 6	1 3	–	3 4	1 2	4 1
	III-4	11 8	1 8	–	5 3	0 9	3 2
024	II-1	11 8	1 8[a]	5 8	4 8	0 9[d]	–
	II-2	11 8	1 8	5 8	4 8	0 9	–
	II-4	11 8	1 8	2 8	4 8	0 9	–
	II 5	11 8/10 6	1 8/1 3	5 8/2 8	5 3/4 8	1 2/0 9	–
	III-1	11 8	1 8	5 8	4 8	0 9	–
	III-2	10 6	1 8	2 8	5 3	1 2	–
025	II-2	11 8/10 6	8 0/6 3[b]	5 8/2 8	4 8/3 9	6 2/1 4[c]	4 1/3 2
	III-1	10 6	8 0	2 8	3 9	6 2	3 2
	III-2	10 6	6 3	2 8	3 9	6 2	3 2
	III-5	10 6	8 0	5 8	4 8	1 4	4 1
	III 7	10 6	6 3	5 8	4 8	1 4	4 1
026	II-2	10 6	1 3[a]	2 8	5 3	1 4[a]	–
	II-4	10 6/10 6	1 8/1 3	5 8/2 8	4 0/3 9	1 4/1 4	–
	II-5	10 6	1 8	2 8	3 9	1 4	–
	II-6	11 8/10 6	1 8/1 8	5 8/2 8	4 0/3 9	1 4/1 4	–
	II-7	11 8	1 8	2 8	3 4	6 2	–
	II 8	11 8	1 8	2 8	3 9	1 4	–
	II-10	10 6/10 6	1 8/1 3	5 8/2 8	5 3/4 0	1 4/1 4	–
	II-11	10 6/10 6	1 8/1 3	5 8/2 8	5 3/4 0	1 4/1 4	–
	III-1	10 6	1 3	2 8/2 8	3 9	1 4	–
	III-3	11 8/10 6	1 8/1 8	5 8	4 0/3 4	6 2/1 4	–
	III 5	10 6	1 8	2 8	3 9	1 4	–
	III-7	10 6	1 3	2 8	5 3	1 4	–
	III-8	10 6	1 3	2 8	5 3	1 4	–
	III 9	10 6	1 8	2 8	5 3	1 4	–
	III-10	10 6	1 8	2 8	5 3	1 4	–
	III-11	10 6	1 8	2 8	5 3	1 4	–
	IV-1	11 8	1 8	5 8	4 0	1 4	–
	IV-2	10 6	1 8	5 8	4 0	1 4	–
027	I-2	11 8/10 6	1 8/1 3[a]	nt	5 3/4 5	1 2/0 9[d]	3 2/3 2
	II-1	11 8/10 6	1 8/1 8	5 8/2 8	5 3/5 3	1 2/0 9	4 1/3 2
	III-1	11 8	1 8	5 8	5 3	1 2	4 1
029	II-1	11 8	1 3[a]	–	4 5	–	4 1
	II-3	10 6	1 8	–	4 5	–	4 1
	II-4	10 6	1 8	–	4 5	–	nt
	II-5	11 8	1 3	–	4 5	–	nt
	II 8	11 8/10 6	1 8/1 3	–	4 8/3 4	–	4 1/3 2
	III-2	10 6	1 3	–	3 4	–	4 1
	III 3	11 8	1 8	–	4 8	–	3 2

Table 3.2 (continued)

Kindred	Individual	Genotypes					
		DXYS1	F9	DXS15	DXS52	F8	DXS134
030	II-4	10 6	1 8[a]	–	5 3	–	–
	II-6	10 6/10 6	1 8/1 3	–	4 8/4 0	–	–
	II-7	10 6	1 8	–	3 9	–	–
	III-1	10 6	1 8	–	4 8	–	–
	III-2	10 6	1 3	–	4 0	–	–
	III-4	10 6/10 6	1 8/1 3	–	4 0/3 9	–	–
	IV-2	10 6	1 3	–	4 0	–	–
046	II-1	10 6	1 8[a]	–	4 5	0 9[d]	3 2
	II-2	11 8/11 8	1 8/1 8	–	4 8/4 5	0 9/0 9	4 1/3 2
	II-3	11 8	1 3	–	4 8	1 2	4 1
	III-1	11 8	1 8	–	4 8	0 9	4 1
	III-2	11 8	1 8	–	4 8	0 9	4 1
	III-3	11 8	1 8	–	4 5	0 9	3 2
047	I-1	nt	nt[c]	–	3 4	–[d]	–
	II 1	11 8/10 6	–	–	4 8/3 4	–	–
	II-2	11 8/11 8	1 8/1 7	2 8/2 8	4 8/3 4	0 9/0 9	–
	II-3	11 8/10 6	–	2 8/2 8	4 8/3 4	0 9/0 9	–
	II-4	11 8/10 6		–	4 8/3 4	–	–
	II-6	11 8/11 8	1 8/1 7	2 8/2 8	4 8/3 4	0 9/0 9	–
	III-1	10 6	–	–	3 4	–	–
	III-2	11 8		–	3 4	–	–
	III-3	11 8	1 8	–	4 8	–	
	III 6	10 6	–	–	3 4	–	–
	III-7	11 8	–	–	3 4	–	–
	III-9	10 6/10 6	1 7/1 7	5 8/2 8	5 3/4 8	0 9/0 9	
	III 11	nt	–	–	3 4	–	–
	III-12	11 8	–	–	4 8	–	–
	III 16	11 8	1 8	–	4 8	–	–
	III 17	11 8	nt	–	4 8	–	–
	III-18	11 8	1 7	–	3 4	–	–
	III 19	11 8	nt	–	3 4	–	–
	III-20	11 8	1 8	–	4 8	–	–
	IV-1	10 6	nt	5 8	5 3	0 9	
	IV-2	10 6	nt	2 8	4 8	0 9	–

[a] *Taq*I polymorphism
[b] *Bam*HI/*Eco*RI polymorphism
[c] *Dde*I polymorphism
[d] *Bcl*I polymorphism
[e] *Kpn*I/*Xba*I polymorphism

Each of the 10 NDI families was informative for at least one of the seven polymorphic DNA markers employed. Probe St14, defining locus DXS52, was informative in nine out of ten families and yielded a total of 36 scorable meioses. The genotypes of all individuals, that are relevant to the analysis, are presented in Table 2. Genotypes for DXS105 are not shown as only one family was informative for this marker.
Two-point linkage data for NDI and the seven DNA markers are summarized in Table 3.

Table 3.3 Linkage relationships for nephrogenic diabetes insipidus (NDI) and marker loci

Marker	No of informative families	Lod scores 0 (recombination fraction)							z^a	θ^b
		0 00	0 01	0 05	0 10	0 20	0 30	0 40		
DXYS1	8	-	−14 58	−7 19	−4 24	−2 10	−0 59	−0 11	0 00	0 50
F9	8	-	−7 35	2 70	−1 14	−0 06	0 29	0 19	0 31	0 32
DXS105	1	0 10	0 09	0 08	0 06	0 02	0 01	0 00	0 10	0 00
DXS15	5	3 02	3 08	2 94	2 50	1 80	1 12	0 47	3 23	0 00
DXS52	9	10 35	10 33	9 44	8 41	6 25	3 96	1 63	10 35	0 00
F8	5	2 19	2 16	1 98	1 75	1 28	0 78	0 34	2 19	0 00
DXS134	5	2 09	2 07	1 89	1 68	1 22	0 75	0 29	2 09	0 00

[a] Maximum likelihood estimation of the lod score
[b] Recombination fraction at $\hat{z}$

There was no evidence of linkage between NDI and the most proximal marker tested DXYS1 (pDP34). Lod scores were uniformly negative for all values of θ. Similarly, close linkage was excluded for the second most proximal marker tested, F9 (pVIII, pXIII, and pVI.3). The maximum combined lod score was 0.29 at θ= 0.30. The θ max between NDI and DXS105 (cX55.7) was not significant. Tight linkage

was found between the gene coding for NDI and the markers DXS15 (DX13), DXS52 (St14), F8 (p114.12 and p482.6) and DXS134 (cpX.67). These markers are located within band Xq28 and are closely linked (Oberlé et al., 1985; Hofker et al., 1987).
No cross-overs were observed between NDI and each of these four markers, giving maximum lod scores of 3.23 (DXS15), 10.35 (DXS52), 2.19 (F8), and 2.09 (DXS134) respectively. Thus, our data are not instructive as regards the localisation of NDI in relation to the Xq28 markers.
As to the DNA markers in the Xq28 region, we found no cross-overs between DXS15 and DXS52. However, in family 023 a recombination event was observed between F8 and DXS52 (DXS15 was not informative in this family); in family 027 a cross-over separated DXS134 from F8 and DXS15 (DXS52 was not informative in this family).

3.5. DISCUSSION

We have observed tight linkage and absence of recombination between the NDI gene and four marker loci, all of which are known to be located at the band Xq28 near the long arm telomere of the X-chromosome (Oberlé et al., 1985; Hofker et al., 1987). In contrast, close linkage could be excluded for DXYS1 and the F9 marker, both of which map further proximal to the Xq and cleary outside the Xq28 region.
These results assign the NDI gene to the distal long arm of the X chromosome. Moreover, the fact that results for all informative families were concordant argues against genetic heterogeneity of this disorder and suggests that the disease is due to a single X-linked gene defect.
The assignment of the NDI gene to Xq28 is consistent with a previous study that has excluded linkage with the Xg blood group, on the distal short arm of the X-chromosome (Bode and Miettinen, 1970). Close linkage of NDI with Xq28 markers is also corroborated by a recent study, which was conducted simultaneously in one large family (Kambouris et al., 1988; see also Davies et al., 1987).

These results place the NDI gene in the vicinity of the genes for haemophilia A (McKusick, 1983), adrenoleukodystrophy (ALD) (Abourg et al., 1987, van Oost et al., 1987), Emery Dreifuss muscular dystrophy (EMD) (Thomas et al., 1986), the Hunter syndrome (Upadhyaya et al., 1986), G-6-PD deficiency and colour blindness (see Fig. 2).

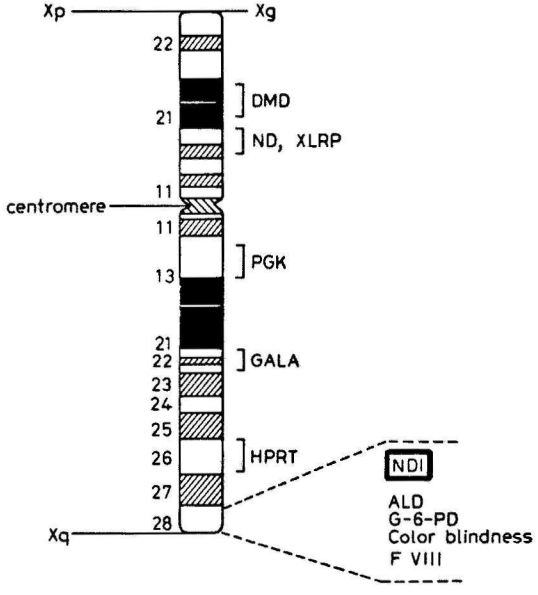

Fig. 3.2: Map of the X chromosome indicating the localization of the NDI gene and various other genes. PGK Phosphoglycerate kinase, GALA α-galactosidase, HPRT hypoxanthineguanine phosphoribosyltransferase.

The precise orientation of the NDI gene relative to the aforementioned gene loci remains to be elucidated. To our knowledge, a combination of any of these disorders with NDI, which might point to a deletion spanning two or more loci, has not been reported to date.

As to the relative order of DNA markers in the Xq28 band, previous

reports have been controversial: Drayna and White (1985), employing multifactor analysis, determined the most likely order in this region as centromere-(DXS52, DXS15)-F8-telomere. The same order was suggested by Mulligan et al. (1987). They identified a recombination between DXS52 and F8 in a family with the fragile X syndrome. Others proposed the order F8-DXS15-DXS52, based on phase-known (Carpenter et al., 1987; Connor et al., 1987; see also Davies et al., 1987), and phase-unknown (Vidaud et al., 1987; Bhattacharya et al., 1987; see also Davies et al., 1987) recombinations or somatic cell hybrids (Tantravahi et al., 1986). Owing to the scarcity of recombination in our families, we could not decide between these two possibilities.

In general, subtelomeric chromosomal regions are characterized by a high incidence of cross-overs, which means that compared to the physical map, the genetic map of these segments appears to be enlarged. Therefore, the observed lack of recombination between NDI and four Xq28 markers suggests that their physical distance is very small. This offers promise for physical mapping and eventually, for the isolation of the NDI gene. Further studies will concentrate on the ordering of genes in this region, employing other genetic markers such as the colour blindness genes as well as long-range restriction mapping (Carle et al., 1986).

The closely linked DNA markers, that have been identified in this study, should be a valuable tool for reliable carrier detection and prenatal diagnosis. Early detection of NDI is important because, if not treated, it frequently leads to brain damage and severe mental retardation.

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CHAPTER 4

THREE-POINT LINKAGE ANALYSIS USING MULTIPLE DNA POLYMORPHIC MARKERS IN FAMILIES WITH X-LINKED NEPHROGENIC DIABETES INSIPIDUS

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THREE-POINT LINKAGE ANALYSIS USING MULTIPLE DNA POLYMORPHIC MARKERS IN FAMILIES WITH X-LINKED NEPHROGENIC DIABETES INSIPIDUS.

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4.1 ABSTRACT

The gene for X-linked nephrogenic diabetes insipidus (NDI), a disorder which, if untreated, causes severe dehydration, mental retardation, and possibly death in affected males, has been mapped recently to the Xq28 band through demonstration of linkage to the DXS52 locus and other DNA markers (N. Knoers et al., 1987, Cytogenet.Cell genet. 46:640; M.Kambouris et al., 1987, Cytogenet.Cell genet. 46:636). Linkage studies in eleven families with NDI have enabled us to map the NDI gene between closely linked flanking markers in the Xq28 region and to obtain the following gene order: centromere-F9-DXS98-F8/CBD,CBP-DXS52/NDI-DXS134-telomere.
These results have implications for presymptomatic and prenatal diagnosis of NDI and should also improve the prospects for identifying the fundamental gene defect underlying the disorder.

4.2 INTRODUCTION

The locus for X-linked Nephrogenic Diabetes Insipidus (NDI) has been assigned to the distal long arm of the human X chromosome, by demonstrating tight linkage to the DNA-marker loci DXS52 (probe St14) (Knoers et al., 1988a), DXS15 (probe DX13), F8 (probes 144.12 and 486.2) and DXS134 (probe cpX67) (Knoers et al., 1988b; Kambouris et al., 1988), all of which are located within band Xq28 (Oberlé et al., 1985; Hofker et al ., 1987). However, until now, the precise location of the disease locus could not be determined, because of the absence of recombination between the NDI gene and any of the aforementioned restriction fragment length polymorphism (RFLP) markers.
In order to define the position of the NDI locus more precisely and to generate a genetic map of the Xq28 region, we have performed multipoint linkage analyses in eleven Dutch families employing six DNA markers from the distal long arm of the X chromosome. The results of these studies are reported in the present communication.

Subjects

Eleven families with NDI from the Netherlands, comprising 20 affected males, were included in the study. Ten of these families have been described previously (Knoers et al., 1988b). Diagnosis of NDI was confirmed by the lack of increase of urine osmolality after infusion of the synthetic vasopressin analogue DDAVP (1-desamino-8-D-arginine-vasopressin). Maximal urine osmolalities that could be achieved after DDAVP administration were 51-198 mosm/kg (normal >805 mosm/kg) .

DNA analysis

The methods used in DNA analysis have been described previously (Knoers et al., 1988a, 1988b). Briefly, DNA was extracted from blood lymphocytes and digested to completion with the appropriate restriction enzyme under conditions recommended by the suppliers. The restriction fragments were separated electrophoretically and transferred to GENE SCREEN PLUS filters (NEN research products, Boston) and [^{32} P]-labeled probes were hybridized to the blots.

Probes

Six cloned DNA probes corresponding to the loci F9, DXS98, DXS134, DXS52, CBD/CBP, and F8 (Table 1) were used in the linkage analysis. Two other markers that were tested, DXS15 (probe DX13) and DXS33 (probe MN12), were not treated as separate loci in the analysis because neither of the two showed recombination with NDI and the DXS52 marker (probe St14). This is in keeping with recent evidence, obtained by long-range physical mapping techniques, that DXS15, DXS52, and DXS33 are physically very close (Patterson et al., 1987).

Linkage analysis

Three-point linkage analysis was performed assuming X-linked recessive inheritance, employing the ILINK programme of the LINKAGE package (Lathrop and Lalouel., 1984), which was kindly provided

by Dr. J. Ott (Columbia University, New York). The frequency of the mutant NDI allele in females was taken as 1 in 10^5 and a mutation rate of 5×10^{-6} was assumed. For linkage analysis, biochemical abnormalities in females were not taken into account.

Table 4.1 X-Chromosome markers used in this study

Locus	Probe	Restriction enzyme	Allele size (kb)	Allele frequency	Genomic location	Ref
F9	pVIII	*Taq*I	1 8	0 71		(5)
			1 3	0 29		
	pXIII	*Dde*I	1 75	0 24	Xq26–q27	(29)
			1 70	0 76		
	pVI 3	*Bam*HI/*Eco*RI	8 0	0 94		(11)
			6 3	0 06		
DXS98	4D8	*Msp*I	25	0 82	Xq26–qter	(3)
			7 8	0 18		
F8	p114 12	*Bcl*I	1 2	0 37		(10)
			0 9	0 63		
					Xq28	
	p482 6	*Kpn*I/*Xba*I	6 2	0 41		(30)
			1 4	0 59		
DXS52	St14	*Taq*I		complex	Xq28	(20)
DXS134	cpX67	*Msp*I	3 7	0 70	Xq28	(12)
			3 4	0 30		
CBD,CBP	hs7	*Sst*I	18 + 7 5	?	Xq22–q28	(19)
			18			
			7 5			

4.4 RESULTS AND DISCUSSION

Table 2 presents the best-supported order and the relative likelihood of alternative orders of all possible three-locus orders of six loci (five DNA markers and the disease locus). The F9 marker is not included in the table because it maps clearly outside the Xq28 region. This was confirmed by three-point analyses indicating that F9 is located proximal to the other six loci (odds > 100:1, data not shown).

Orders with a posterior probability of less than 0.01 were taken as

Table 4.2 Best-supported order of loci and relative likelihoods for the alternative orders of all possible three-locus orders involving DXS98, F8, DXS134, CBD, CBP, DXS52, and the NDI gene.

Best supported order of loci (a, b, c)	Respective % recombination between adjacent loci	Relative likelihoods of alternative orders	
		b-a-c	a c-b
NDI-DXS52-F8	0 001-0 058	1	$6\,3 \times 10^{-3}$
DXS134-NDI-F8	0 001-0 092	$9\,7 \times 10^{-1}$	$3\,1 \times 10^{-1}$
DXS134-NDI-DXS52	0 046-0 001	$4\,0 \times 10^{-1}$	1
CBD,CBP-NDI-DXS52	0 015-0 001	$1\,6 \times 10^{-2}$	1
NDI-CBD,CBP-F8	0 021-0 001	$1\,0 \times 10^{-2}$	$7\,9 \times 10^{-1}$
CBD,CBP-NDI-DXS134	0 030-0 021	$6\,3 \times 10^{-1}$	$8\,1 \times 10^{-1}$
DXS98-NDI-DXS52	0 115-0 001	$8\,0 \times 10^{-2}$	1
DXS98-NDI-F8	0 001-0 071	1	$1\,9 \times 10^{-1}$
DXS98-NDI DXS134	0 001-0 101	$9\,6 \times 10^{-1}$	$4\,9 \times 10^{-1}$
DXS98-NDI-CBD,CBP	0 064-0 039	$6\,4 \times 10^{1}$	$4\,0 \times 10^{-1}$
DXS134-DXS52-F8	0 025-0 026	$6\,2 \times 10^{-1}$	$5\,2 \times 10^{-2}$
CBD,CBP-DXS52-DXS134	0 001-0 046	1	$5\,0 \times 10^{3}$
DXS52-CBD,CBP-F8	0 001-0 023	1	$7\,7 \times 10^{-1}$
DXS98-DXS52-F8	0 129-0 062	$3\,0 \times 10^{-1}$	$1\,8 \times 10^{1}$
DXS98-DXS52-DXS134	0 420-0 580	$1\,9 \times 10^{-2}$	$1\,5 \times 10^{-1}$
DXS98-DXS52-CBD,CBP	0 115-0 001	$1\,9 \times 10^{-6}$	$7\,9 \times 10^{1}$
F8-CBD,CBP-DXS134	0 001-0 112	$9\,8 \times 10^{-1}$	$4\,7 \times 10^{-2}$
DXS134-CBD,CBP-DXS98	0 044-0 108	$1\,3 \times 10^{-1}$	$3\,1 \times 10^{-2}$

rejected. Comparison of all possible of seven locus orders (including F9) indicated that all except two, given below ,encompassed at least one rejected three-locus order:

(1) F9 - DXS98 - F8/ CBD,CBP - DXS52/ NDI - DXS134

(2) F9 - DXS98 - DXS134 - DXS52/NDI - F8/CBD,CBP.

Analysis of individual cross-over events strongly favored possibility 1. Consistently, the gene order F9 - DXS98 - F8/CBD,CBP-DXS52/NDI -DXS134 requires the least number of recombinants to explain segregation patterns observed, as exemplified in Fig.1. This map is in perfect agreement with the consensus gene order put forward during the most recent Human Gene Mapping conference (HGM9,

Davies et al., 1987): Xcen - F9 - DXS105 - DXS98 - F8 -DXS33- DXS52 - DXS15 - DXS134 - qter.

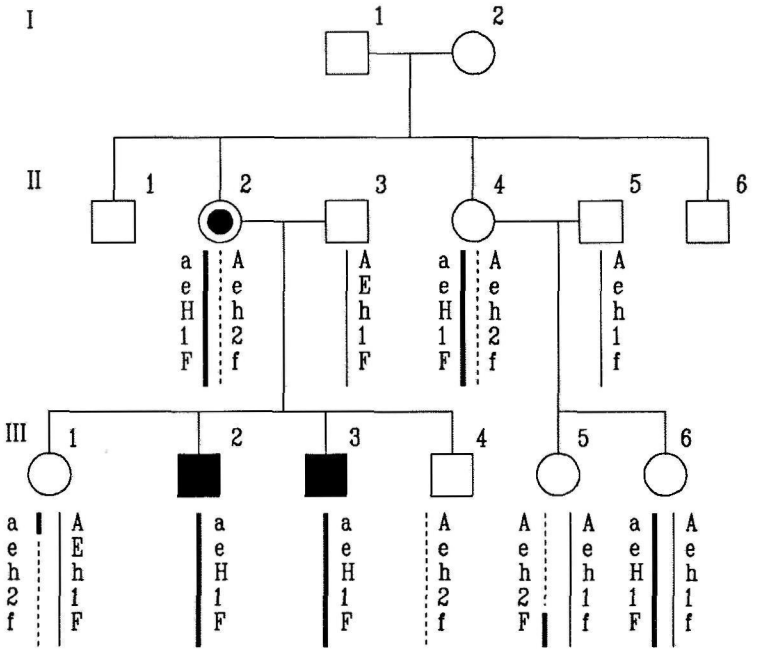

Fig. 4.1: Pedigree of a family with X-linked nephrogenic diabetes insipidus using five polymorphic DNA markers on Xq (DXS98/p4D8:A,a; F8/p144.12: E,e; CBD,CBP/phs7: H,h;DXS52/pSt14:1,2; DXS134/ cpX67: F,f) ■ affected males; ◉ obligatory carrier.

Taken together, the results indicate that the NDI gene maps in the close vicinity of the markers DXS52, DXS33 and DXS15. Moreover, these analyses provide us with closely linked flanking markers for the NDI gene: F8 as well as CBD,CBP on one and DXS134 on the other side of the disease locus. This finding should greatly increase the reliability of both carrier detection and early (prenatal) diagnosis of NDI. Early detection is a prerequisite for early treatment of the disease, which can prevent brain damage and severe mental retardation.

In addition, the refined genetic mapping achieved in this study should improve the prospects for cloning the NDI gene, because, compared to is physical length, the subtelomeric Xq region is characterized by a high rate of recombination. Given the tight linkage between NDI and several Xq28 markers, this implies that the corresponding physical distances may be very small. This, as well as the available unique collection of NDI patients, offers promise for the detection of structural aberrations, such as small deletions, employing Southern blotting and pulsed-field gel electrophoresis with DNA probes from the F8-DXS134 interval.
Submicroscopic deletions have been detected in a variety of X-linked disorders (Wieringa et al., 1985; Gal et al., 1985; Cremers et al., 1987) and have paved the way for the molecular isolation of several genes (Monaco et al., 1986; Royer-Pokora et al.,1986; Page et al., 1987).

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CHAPTER 5

FIBRINOLYTIC RESPONSES TO 1-DESAMINO-8-D-ARGININE-VASOPRESSIN IN PATIENTS WITH CONGENITAL NEPHROGENIC DIABETES INSIPIDUS.

Nephron, 54, 1990, 322-326

FIBRINOLYTIC RESPONSES TO 1-DESAMINO-8-D-ARGININE VASOPRESSIN IN PATIENTS WITH CONGENITAL NEPHROGENIC DIABETES INSIPIDUS

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5.1. ABSTRACT

Fibrinolytic responses to infusion of 1-desamino-8-D-arginine-vasopressin (DDAVP) were assessed in 6 males with congenital Nephrogenic Diabetes Insipidus (NDI), 6 carriers of the NDI gene and 6 normal control subjects. Tissue-type plasminogen activator (t-PA) activity and antigen increased significantly in normal subjects, while plasminogen activator inhibitor (PAI) activity decreased. None of these changes were observed in patients with NDI. In 2 female carriers normal fibrinolytic responses were seen, while in the other carriers responses were delayed. These findings are consistent with the concept of a general V_2-receptor defect in congenital NDI. DDAVP tests are of limited use in NDI carrier detection.

5.2. INTRODUCTION

The neurohypophyseal hormone arginine vasopressin (AVP) exerts its effects through stimulation of at least two pharmacologically distinct classes of receptors: The pressor response of this hormone and other actions, such as glycogenolysis and platelet aggregation, are mediated via vascular (V_1) receptors, while the antidiuretic effect is mediated via renal tubular (V_2) receptors. V_2 vasopressin receptors are linked to a membrane adenylate cyclase, whereas binding of vasopressin to V_1 receptors has been shown to stimulate phosphatidylinositol breakdown and Ca^{2+} mobilization. Apart from differences in functional properties, V_1 and V_2 receptors can be distinguished by their vasopressin analogue recognition pattern [1-2].

1-desamino-8-D-arginine vasopressin (DDAVP or Desmopressin) is a synthetic derivative of the native hormone which has potent antidiuretic V_2-activity but, unlike vasopressin and other analogues, is virtually free of stimulating effects on blood pressure [3]. These differences are the result of molecular modification of vasopressin involving deamination of hemicysteine at position 1

which results in a more prolonged duration of antidiuretic action, and substitution of the D-isomer of arginine for L-arginine at position 8 which results in a diminished pressor activity. In contrast, DDAVP exerts a considerable vasodilatory action, manifested by flushing, a fall in diastolic blood pressure and a rise in pulse rate [4].

Other extrarenal effects of DDAVP have been described recently: The drug provokes a marked, transient, release of factor VIII coagulant activity (FVIII:C) and von Willebrand factor antigen (vWF:Ag) from endothelial storage sites [5]. Because of this property DDAVP is well established in the treatment of mild and moderate hemophilia A and type I von Willebrand's disease [6-9].

In addition, DDAVP causes a significant dose-dependent increase in fibrinolytic activity and this has been attributed to a rapid release of extrinsic (tissue-type) plasminogen activator (t-PA) into the circulation [10]. The mechanisms by which desmopressin causes vasodilatation and increases in FVIII components and t-PA levels are still incompletely understood.

Patients with X-linked Nephrogenic Diabetes Insipidus (NDI) are resistant to the antidiuretic action of vasopressin and DDAVP [11], due to a defect either in the renal V_2-receptor itself or in the process beyond the intracellular formation of cyclic-AMP [12]. Kobrinsky et al. [13] have demonstrated absence of FVIII:C and vWF:Ag responses to DDAVP in 2 NDI patients. From this observation, they concluded that the action of DDAVP on haemostasis is mediated through stimulation of extrarenal V_2-receptors. Recently, this assumption was supported by the investigation of Bichet et al. [14], showing absence not only of FVIII release, but also of haemodynamic effects in 7 NDI patients after administration of desmopressin. They speculated that the interaction of desmopressin with these extrarenal V_2-receptors could be responsible for both the vasodilatory effects and the increase in FVIII components. t-PA levels were not quantified in these studies. These findings have prompted us to evaluate fibrinolytic parameters, such as t-PA antigen, t-PA activity and the recently discovered plasminogen activator inhibitor (PAI) [15] in NDI patients and healthy controls following infu-

sion of DDAVP. In addition, we have measured DDAVP-induced fibrinolytic responses in NDI carrier females.

5.3. MATERIAL AND METHODS

Subjects

Our study included 6 patients with X-linked recessive NDI, 6 female NDI carriers and 6 healthy control individuals. Approval of the Medical Ethical Commission of the hospital was obtained for this investigation. In the patients, aged 8, 15, 16, 22, 25 and 26 years, respectively, diagnosis of NDI was based on clinical symptoms and the lack of increase of urine osmolality after administration of DDAVP. Maximal urine osmolalities that could be achieved after DDAVP infusion were 51-198 mosm/kg (normal >805 mosm/kg). The pedigrees of the 6 patients have been described elsewhere [16]. All patients discontinued hydrochlorothiazide medication 24 hours before the study.

Heterozygote female carriers (43-54 years old) were identified by DNA linkage analysis with various DNA markers known to be closely linked to the NDI gene on the subtelomeric region of the long arm of the X chromosome [16,17].

The control group consisted of 6 healthy volunteers, 2 women and 4 men (24-28 years old), who had agreed to participate in the experiment and were fully aware of the purpose of the investigation. The women were not using oral contraceptives, which are known to affect t-PA levels [18].

DDAVP administration

Throughout the experiment DDAVP tests were always performed in the morning between 9.00 and 11.00 a.m. to avoid the influence of the normal daily fluctuation of fibrinolytic activity [19].

The DDAVP (Minrin ^{R}) was supplied by Ferring Pharmaceuticals AB (Malmö, Sweden). A dose corresponding to 0.3 μg DDAVP/kg body weight was diluted in 100 ml saline and was injected slowly over a period of 10 min into a cubital vein of the recumbant subject, 1

hour after his confinement to bed. A 4.5 ml blood sample was collected from the indwelling venipuncture 10 min before, immediately before and 10, 20, 30, 60 min after the start of the infusion. Systolic and diastolic blood pressure and heart rate were monitored at 3-min intervals during and 15 min after the study.

Blood samples

Blood samples were transferred into plastic tubes, containing 0.5 ml 3.8% sodium citrate, and cooled immediately on melting ice. Platelet-poor plasma, obtained by centrifugation at 4° C for 10 min at 3,000g, was quickly frozen in aliquots of 1.5 ml and stored at -70° C until assays were performed.

Assay methods

t-PA antigen was quantified by an enzyme immunoassay [20]. t-PA-activity was measured in an amidolytic assay system exactly as described by Verheyen et al. [21], using a chromogenic plasmin substrate, H-D-Val-Leu-Lys-p-nitroanilide (S2251 from Kabi Vitrum, Stockholm, Sweden), human lys-plasminogen and soluble fibrin fragments prepared by digestion of human fibrinogen (grade L, Kabi Vitrum, Stockholm, Sweden) with cyanogen bromide. This assay is based on t-PA mediated conversion of plasminogen to plasmin , which in turn cleaves an oligopeptide-nitro-anilidine substrate. The p-nitroaniline released is measured spectrophotometrically. PA-inhibitor was determined by titration of 1:20 fold diluted plasma with stepwise increasing amounts of purified t-PA [21].

Statistical methods

Separate for each time point the method analysis of variance was applied, based on logarithmic transformed data, and a mutual comparison was done using the method of Tukey [22].

5.4. RESULTS

The fibrinolytic responses to DDAVP obtained in control subjects,

Fig.5.1 : t-PA activity response to DDAVP infusion in normal controls (———) and NDI patients (- - -). Values at 0 min are expressed as 100%. Bars indicate 1 ± SE.

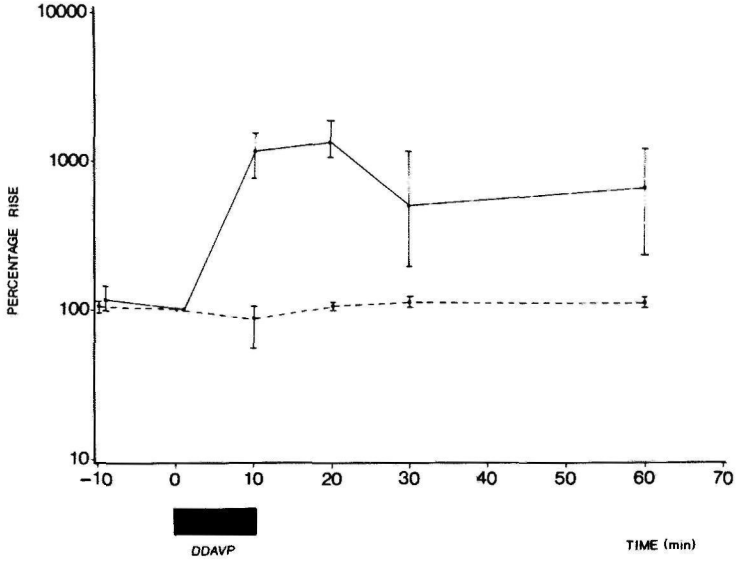


Fig.5.2 : t-PA antigen response to DDAVP infusion in normal controls (———) and NDI patients (- - -). Values at 0 min are expressed as 100%. Bars indicate 1 ± SE.

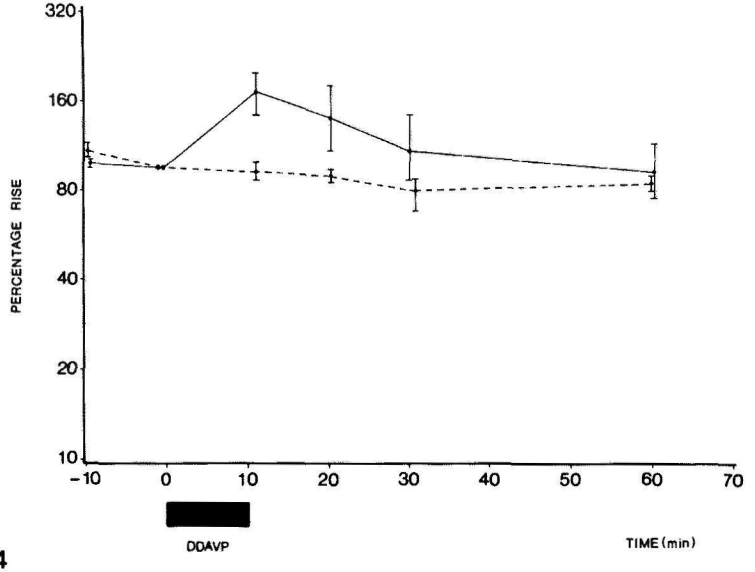


Fig.5.3 : t-PA inhibitor activity response to DDAVP infusion in normal controls (———) and NDI patients (- - -). Values at 0 min are expressed as 100%. Bars indicate 1 ± SE.

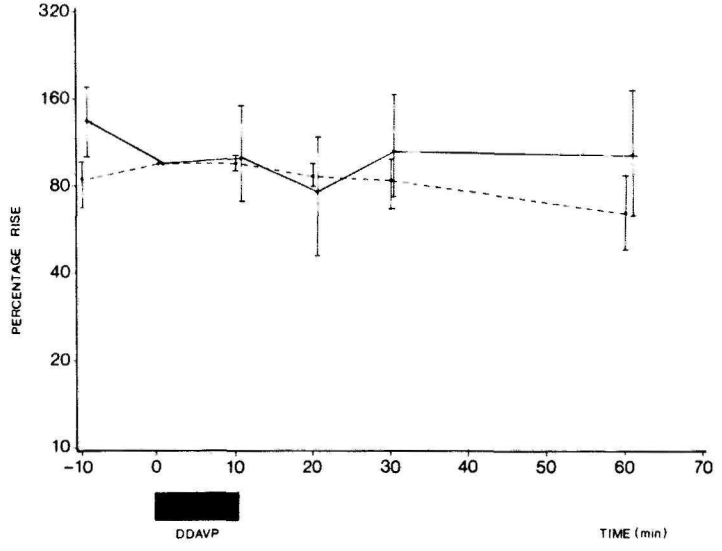
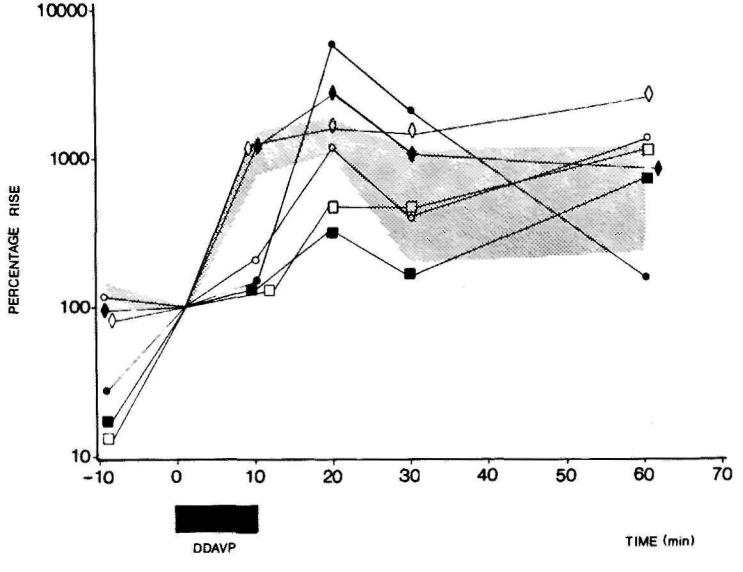


Fig.5.4 : t-PA activity response to DDAVP infusion in 6 female NDI carriers. Values at 0 min are expressed as 100%. Normal range (as derived from Fig.1) is indicated.



NDI patients and carrier females are shown in Fig.1 for t-PA activity, in Fig.2 for t-PA antigen and in Fig.3 for t-PA inhibitor (PAI) activity. Results are expressed as percentages of baseline values. In normal subjects the infusion of DDAVP produced a 15-fold rise ($p < 0.05$) in t-PA activity and a 2-fold rise in t-PA antigen ($p < 0.05$) within 10 min, while PAI activity decreased slightly within 20 min. In contrast, none of the patients with congenital NDI showed any significant change in t-PA activity, t-PA antigen, and PAI values. The fibrinolytic responses in the 6 female NDI carriers were rather diverse as exemplified in Fig.4 for t-PA activity. After 10 min, 2 carriers showed a normal increase in t-PA activity, while the other 4 demonstrated blunted responses, with the mean response for all carriers amounting to 40 % of mean control values. After 20 min the majority of carriers showed t-PA levels in the range of normal conrols.

Comparison between patients and normal subjects indicated that t-PA activity responses in patients with NDI were significantly ($p<0.05$) different from those in normal subjects at each sampling time. 10 min after DDAVP injection t-PA antigen values in NDI patients were significantly ($p<0.05$) different from those in normal control individuals.

PAI levels did not significantly differ between the two groups at any sampling time.

DDAVP infusion was followed by a decrease in diastolic blood pressure and a significant rise in pulse rate in all normal individuals but in none of the NDI patients (data not shown).

Flushing was observed in some, but not in all normal subjects, but was absent in all NDI patients. No other side effects to the drug were experienced. Haemodynamic responses in female carriers varied widely (data not shown).

5.5 DISCUSSION

Nephrogenic diabetes insipidus is an X-linked recessive disease, characterized by polyuria, polydipsia, severe dehydration, fever,

anorexia and failure to thrive. These clinical features are attributed to unresponsiveness of the distal renal tubule to the V_2 receptor-mediated antidiuretic effect of endogenous and exogenous vasopressin. V_1 receptors seem to be intact in NDI patients, since they have normal pressor responses to vasopressin [23].

The present study shows that, in contrast to normal individuals, patients with NDI do not show increased fibrinolytic activity after administration of the specific V_2 agonist DDAVP. This lack in response is due to absence of t-PA release from the vascular endothelium and cannot be attributed to a high level of fast-acting t-PA inhibitor (PAI), since in the majority of NDI patients, t-PA inhibitory capacities in plasma were found to be in the normal range.

Our results indicate that the DDAVP-induced increases in t-PA levels after DDAVP infusion, as well as the previously described elevation of FVIII components and the vasodilatatory effects, are mediated through stimulation of extrarenal V_2 receptors.

At present, the exact location of these extrarenal DDAVP-responsive V_2 receptors remains to be elucidated. There is little evidence to suggest that these receptors are situated on the endothelium itself and are stimulated directly by DDAVP. For instance, no significant release of FVIII:C and vWF:Ag is seen after addition of DDAVP to cultured endothelial cells [24,25] or direct perfusion of isolated human vessels [26]. On the other hand, it has been suggested that these receptors may be located in the central nervous system. Cash et al. [27] hypothesized that vasopressin-like molecules may be humoral messengers acting on a cephalic centre, which may in turn release a second messenger that stimulates the endothelial cells indirectly. However, this putative second messenger has not been identified in bovine pituitary and hypothalamic extracts [28]. In addition, patients with hypopituitarism resulting from surgical adenohypophysectomy respond normally to desmopressin [29,30]. In a study with anephric patients, it was demonstrated that a functioning kidney is not required for the effect of DDAVP on the fibrinolytic activity of the blood [31].

Notwithstanding our present ignorance about the exact cellular site

of the DDAVP receptors, the observation that in NDI patients the V_2 agonist DDAVP does not affect diuresis, or induce haemodynamic [14], haemostatic [13,14] or fibrinolytic (this study) changes supports the concept of a general V_2 receptor defect in this disease.

In female carriers, Kobrinsky et al.[13] have found up to 50 % of the increase in FVIII:C and vWF:Ag after administration of DDAVP in 3 NDI carriers (as expected assuming random X inactivation in female individuals) and suggested that DDAVP may be of help in identifying NDI carriers in families at risk. In our study, we also found a diminished fibrinolytic response in NDI carriers 10 min after DDAVP administration. However, after 20 min the majority of carriers demonstrated responses similar to normal controls. This delayed increase in plasma t-PA levels is most likely due to a reduced amount of functional V_2 receptors in those NDI carriers as there is no evidence that t-PA synthesis or storage in NDI is different from normal. In general, our results are in agreement with those of Kobrinsky et al. However, it is clear that in 2 carriers normal responses were obtained. This means that in some cases identification of carriers by means of DDAVP infusion might be misleading.

Recently, the localization of the gene responsible for NDI has provided us with closely linked markers, which have been shown to be of use for reliable identification of carriers [16,17].

In summary, we have investigated the fibrinolytic responses to DDAVP in patients with X-linked recessive Nephrogenic Diabetes Insipidus, heterozygote female carriers of the NDI gene and normal control individuals. We observed no fibrinolytic responses to DDAVP in NDI patients and normal responses in some carriers. Therefore, DDAVP tests appear to be of limited use for the detection of female NDI carriers. Our results suggest that the fibrinolytic effects exerted by DDAVP, in addition to the previously described haemodynamic and coagulation responses, depend on extrarenal V_2 receptor activation.

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CHAPTER 6

A NEW VARIANT OF NEPHROGENIC DIABETES INSIPIDUS: V_2 RECEPTOR ABNORMALITY RESTRICTED TO THE KIDNEY.

Submitted for publication.

A NEW VARIANT OF NEPHROGENIC DIABETES INSIPIDUS: V_2 RECEPTOR ABNORMALITY RESTRICTED TO THE KIDNEY.

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6.1 ABSTRACT

In congenital Nephrogenic diabetes insipidus (NDI) blunted responses of plasma factor VIII, von Willebrand factor, and plasminogen activator to the synthetic V_2 analogue 1-desamino-8-D-arginine vasopressin (DDAVP) have been reported. In addition, vasodilatory responses to DDAVP appear to be absent in NDI. We describe a boy, who presented shortly after birth with the typical features of NDI, but who showed normal coagulation, fibrinolytic and vasodilatory responses to DDAVP. We conclude that in this patient the defect is confined to the kidney, while in other NDI patients there may be a general V_2 receptor abnormality. These findings point to heterogeneity in NDI.

Key words: Nephrogenic Diabetes Insipidus - Heterogeneity

Abbreviations: NDI= nephrogenic diabetes insipidus; c-AMP = cyclic adenosine monophosphate; DDAVP = 1-desamino-8-D-arginine vasopressin; vWF:Ag = von Willebrand factor antigen; FVIII:C = factor VIII coagulant activity; t-PA = tissue-type plasminogen activator; DNA = deoxyribonucleic acid; RFPL = restriction fragment length polymorphism

6.2 INTRODUCTION

Nephrogenic diabetes insipidus (NDI) is a rare congenital disease, in which the kidneys are resistant to the antidiuretic action of both endogenous and exogenous vasopressin. As a consequence the kidney loses its ability to concentrate urine. The disease is transmitted as an X-linked recessive trait [3,31] although other modes of inheritance have been described [25]. Recent studies have mapped the NDI gene to the distal long arm of the human X chromosome on the basis of linkage analysis of affected kindreds with restriction fragment length polymorphisms (RFLPs) [14,15].
The main clinical features of the disease are dehydration in infan-

cy and early childhood before the autonomy of drinking is established and polydipsia in later life. Some patients with NDI are mentally retarded probably due to severe dehydration and hyperelectrolytemia early in infancy.
The molecular pathogenesis of the disease is unknown. The antidiuretic effect of vasopressin and its synthetic analogue 1-desamino-8-D-arginine vasopressin (DDAVP) is mediated through c-AMP dependent receptors (so-called V_2 receptors) in the distal renal tubule. Theoretically, the defect could be located in the V_2 receptor itself, somewhere between the receptor and the production of c-AMP, or in the process beyond the intracellular formation of c-AMP.
In addition to mediating antidiuresis, DDAVP induces several extrarenal effects. It provokes a marked transient release of von Willebrand factor antigen (vWF:Ag), factor VIII coagulant activity (FVIII: C) and plasminogen activator [7] from endothelial storage sites and exerts a vasodilatory action manifested by facial flushing, a fall in diastolic blood pressure and a rise in pulse rate [6]. Recent studies have demonstrated that patients with NDI do not show these changes in haemodynamic [2], coagulation [2,17] and fibrinolytic [16] parameters after administration of DDAVP. Contrasting with these observations, the study is presented of a 4 year old boy with severe NDI, who had normal vWF:Ag-, plasminogen-activator- and vasodilatory responses to DDAVP. These contrasting observations argue for heterogeneity in NDI.

6.3 CASE REPORT

The patient J.B., a Caucasian male infant, was born after a full-term pregnancy by ceasarian section, carried out because of lack of progress of labour. He was the second child of unrelated parents. Birth weight was 3590 g. His postnatal course was complicated by hyperbilirubinemia, for which he received phototherapy for two days. He was discharged on the 10th day of life, having regained his birth weight. At the age of 2 months he was readmitted to the hospital because of failure to gain weight normally, constipation,

dehydration and fever, which had persisted since the age of two weeks. On admission a dystrophic, slightly dehydrated child was seen with weight just 600 g above the birth weight. Serum sodium was 170 mmol/l, chloride 130 mmol/l and daily urine output ranged from 1000 to 1400 ml with very low urine osmolality (<70 mosmol/kg). Proteinuria, glycosuria and hyperaminoaciduria were absent. Persistance of a constantly low, fixed urinary osmolality together with continuing hypernatremia suggested the diagnosis of NDI. The diagnosis was confirmed by the lack of increase of urinary osmolality after administration of DDAVP. Additional measurement of urinary cAMP levels before and after DDAVP (20 μg, intranasally) administration revealed that the average increment was from 5.87 to 7.02 nmol/μmol creatinine, that is, only 119 % rise from baseline value.

There was no history of diabetes insipidus in the family. DNA analysis with the probe St14, that defines the DNA marker DXS52, which has previously been shown to be closely linked to the NDI gene [14], showed that J.B. had inherited the same St14 allele from his mother as his healthy brother. This St14 allele came from the maternal grandfather.

After informed consent had been obtained from both parents, a DDAVP infusion test was performed to evaluate extrarenal responses to DDAVP.

6.4 METHODS

48 hours before the study the patient discontinued all medication. Food and water were not restricted either before or during the test. After one hour recumbancy, a dose of 0.3 μg DDAVP (Minrin, Ferring Pharmaceuticals, Malmö, Sweden)/kg body weight was diluted in 100 ml saline and was injected slowly over a period of 10 min into a cubital vein.

Systolic and diastolic blood pressure and heart rate were monitored at 3-min intervals during the first 25 min and at 10 min intervals thereafter. A 4.5 ml blood sample was collected from the indwelling

venipuncture 10 min before, immediately before and 10, 20, 30, and 60 min after the start of the infusion. Blood samples were transferred into plastic tubes containing 0.5 ml 3.8% sodium citrate, and cooled immediately on melting ice. Platelet poor plasma, obtained by centrifugation at 4°C for 10 min at 3,000g, was quickly frozen in aliquots of 1.5 ml and stored at -70°C until assays were performed. vWF:Ag was measured by an enzyme-linked immunosorbent assay from Boehringer, Mannheim, Germany. Tissue-type plasminogen activator (t-PA) antigen was quantified by an enzyme immunoasssay according to Rijken et al. [24]. t-PA activity was measured by an amidolytic assay system as described by Verheyen et al.[30].

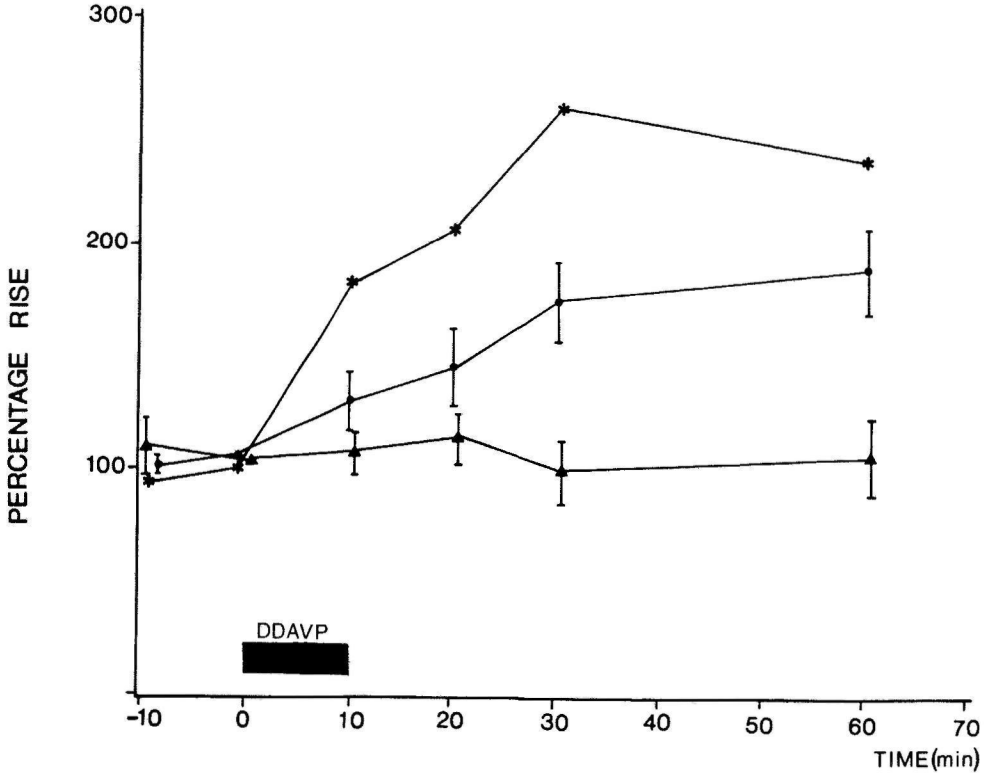

Fig. 6.1 : vWF:Ag response to DDAVP in patient J.B. (*——*), normal control subjects (●——●) and 6 other NDI patients (▲——▲). Results at 0 min are expressed as 100 %. Bars indicate 1 ± S.E.

All data were compared with those obtained in 6 other NDI patients (aged 8-24 years) and in 6 control subjects (aged 24-28 years). The significance of changes during the study were assessed by analysis of variance.

6.5 RESULTS

The responses to DDAVP obtained in J.B., 6 other NDI patients and in 6 normal controls are depicted in Fig.1 for vWF:Ag, in Fig.2 for t-PA activity and in Fig.3 for t-PA antigen. In normal subjects administration of DDAVP caused a 1.5- to 2-fold rise in vWF:Ag within

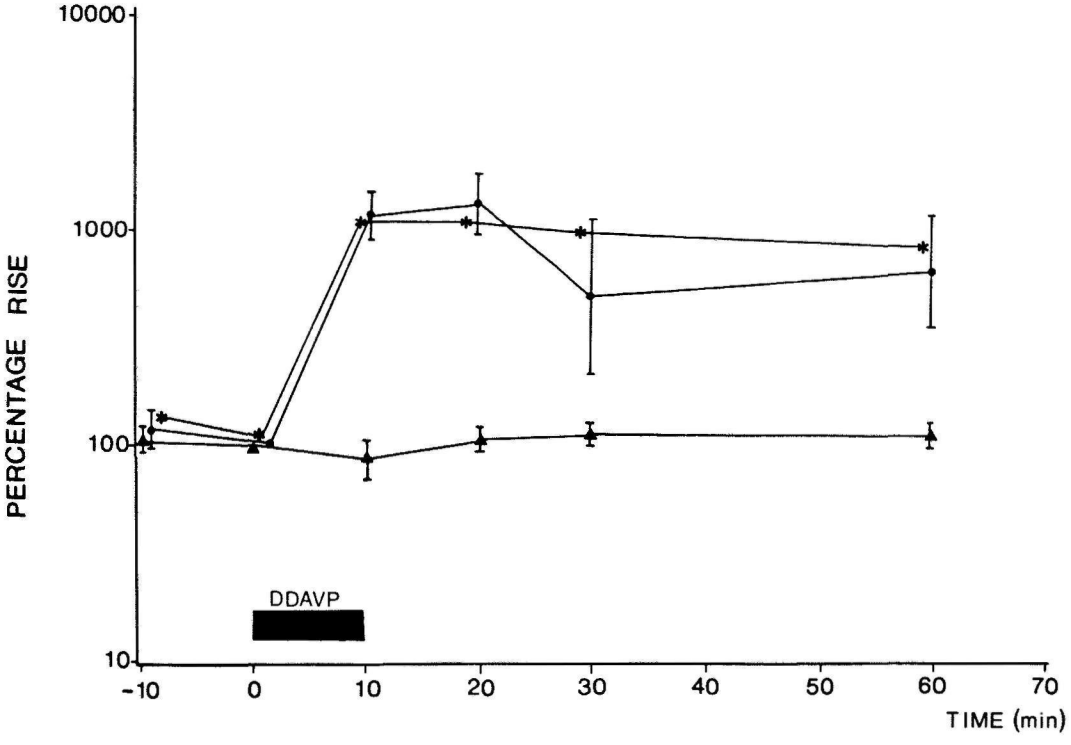

Fig. 6.2: t-PA activity response to DDAVP in patient J.B. (*——*), normal control subjects (●——●) and 6 other NDI patients (▲——▲). Results at 0 min are expressed as 100 %. Bars indicate 1 ± S.E.

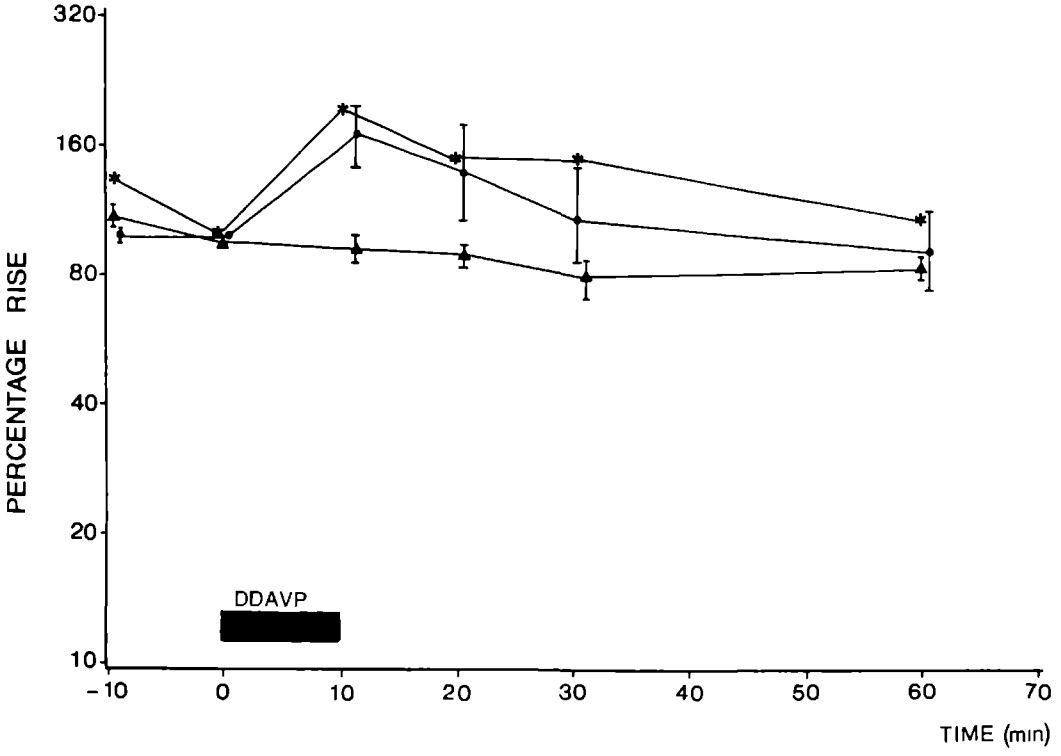

Fig. 6.3: t-PA antigen response to DDAVP in patient J.B (*——*), normal control subjects (●——●) and 6 other NDI patients (▲——▲). Results at 0 min are expressed as 100 %. Bars indicate 1 ± S.E.

30 min, a 15-fold rise in t-PA activity within 10 min and a two-fold rise in t-PA antigen within 10 min. All changes were significant ($p<0.05$). Similar responses were seen in patient J.B. vWF:Ag increased by 200 % within 20 min, t-PA activity by 1309% within 10 min and t-PA antigen by 219% within 10 min. In contrast, none of the other 6 patients showed any significant change in vWF:Ag levels, t-PA activity and t-PA antigen values.

DDAVP infusion was followed by a significant decrease in diastolic blood pressure (10-15%) and a significant rise in pulse rate (15-20%) in all normal individuals. Similar results were obtained in

J.B., while no haemodynamic changes were seen in any of the other NDI patients. Flushing was observed in some, but not in all, normal subjects, and in patient J.B., but was absent in the 6 other NDI patients.

6.6 DISCUSSION

The antidiuretic effect of vasopressin and its synthetic analogue DDAVP is mediated by renal c-AMP dependent vasopressin receptors. These receptors have been classified as V_2 receptors to distinguish them from the calcium dependent V_1 receptors, which mediate the pressor response to vasopressin [11,12]. In addition to mediating antidiuresis, an apparant V_2-mediated action of DDAVP causes release of vWF:Ag, FVIII:C, and plasminogen activator [7] from endothelial storage sites and exerts a vasodilatory action in normal individuals [6]. Recent studies have shown that NDI patients do not show these extrarenal responses to DDAVP [2,16,17].
Based on these observations it was suggested that there may be a general V_2 receptor defect in this disease. The patient presented in this report is of particular interest because he had normal vWF:Ag-, plasminogen activator- and vasodilatory responses to DDAVP administration. In contrast to the blunted responses observed in our other NDI patients, the extent of the response in J.B. was similar to those in the 6 normal subjects studied. Therefore, he does not have a generalized V_2 receptor abnormality.
There is no doubt that he has vasopressin-resistant diabetes insipidus. The clinical symptoms, the fixed low urinary osmolality and the impaired antidiuretic response to DDAVP in the absence of other renal dysfunctions are all in keeping with the diagnosis.
Similar patients have been reported. Brenner et al. [5] described 4 patients, who showed normal plasma factor VIII and vWF responses to DDAVP administration. Two of his patients were females and it cannot be excluded that these 'patients' were in fact carriers of the NDI gene. Similarly, Moses et al. [20] described a 25-year old woman with polyuria and polydipsia and resistance to the antidiu-

retic action of vasopressin, who had significant vWF-, FVIII-, plasminogen activator- and vasodilatory responses to DDAVP. Ohzeki et al. [23] reported on a 20-day old Japanese male infant with congenital NDI, who demonstrated elevation of FVIII:C and vWF levels after administration of arginine vasopressin.

Several explanations for the divergent renal and extrarenal responses to DDAVP in our patient can be offered.

In previous studies, NDI has been divided into two different types (type 1 and type 2) in respect to urinary c-AMP responsiveness [22,32]. In type I NDI, cAMP excretion showed no definite change (<127 % as compared to a 200%-250 % rise in patients with vasopressin-sensitive diabetes insipidus) after stimulation with DDAVP (22). DDAVP produced significant increases (mean 1296%) in urinary cAMP levels in other cases of NDI, which were diagnosed as type 2 of the disease (22, 32). In type I, a defect in the production of c-AMP in the renal tubular cells has been considered, and in the other type in the reception of the c-AMP signal. Although the finding of normal extrarenal responses to DDAVP in our patient could be consistent with a post c-AMP defect being the cause of his renal resistance to vasopressin, this possibility is unlikely because our patient showed only a 119% rise in urinary c-AMP levels in response to DDAVP, without an increase in urinary osmolality. It must, however, be admitted that in animal experiments an increase of cAMP in medullary tissue is not always reflected by an increase in the urinary concentration of cAMP [1].

It seems improbable also that the defect resides at the level of the guanine nucleotide regulatory protein (G-protein) that serves to couple the receptor to the catalytic moiety of the adenylate cyclase complex [28]. In that case, one would expect our patient, in analogy with patients suffering from pseudohypoparathyreoidism type I [26,27], to be resistant to multiple hormones, whose actions are mediated by c-AMP. However, the possibility of a defective G-protein being the cause of the renal unresponsiveness to vasopressin in our patient can not be excluded completely. Although as yet it is thought that the G-proteins involved in adenylate cyclase stimulation (G_s proteins) are 'house-keeping ' G-proteins, that

are expressed in almost every cell [18,21], it seems possible, theoretically, that the G-protein implicated in the action of vasopressin in the distal renal tubule is tissue-specific and differs from the one that couples the extrarenal V_2 receptors to the adenylate cyclase system. Tissue-specificity of G-proteins has already been demonstrated for transducin, a pertussis toxin-sensitive G-protein, involved in visual transduction in photoreceptor cells [19].

It is more likely, however, that the defect is located in the receptor itself. In that case, the finding of blunted renal but normal extrarenal responses to DDAVP could be explained by the hypothesis that the antidiuretic receptor is different from the extrarenal V_2 receptors. Conceivably, in our patient only the renal V_2 receptor is involved in the defect, while in most other NDI patients there is a general V_2 abnormality.

At present the exact site of these extrarenal V_2 receptors remains to be elucidated. It is unlikely that the receptors are located on the endothelium itself, since there is no direct action of DDAVP on the endothelial cell [4,29]. Speculations that these V_2 receptors are situated in the central nervous system and that DDAVP may act through release of a putative second messenger [8], have not been confirmed [9,13].

The finding of normal extrarenal responses to DDAVP in some NDI patients (including our patient), but absence of these responses in others, may reflect phenotypical heterogeneity in NDI. Whether there is genetic heterogeneity also can not be decided from our data. In recent studies the gene for X-linked NDI has been assigned to distal Xq by demonstrating tight linkage to the Xq28 marker DXS52, which is defined by probe St14, and to other distal Xq markers [14,15]. We found that both our patient and his healthy brother had inherited the same St14 allele from their mother. This St14 allele came from the maternal grandfather. Since our patient is a sporadic case, these observations are inconclusive as to whether the disease is due to a mutation in another gene, which is not located at distal Xq, or due to a new mutation in the gene located at Xq28.

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CHAPTER 7

AMILORIDE-HYDROCHLOROTHIAZIDE VERSUS INDOMETHACIN-HYDROCHLOROTHIAZIDE IN THE TREATMENT OF NEPHROGENIC DIABETES INSIPIDUS

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AMILORIDE-HYDROCHLOROTHIAZIDE VERSUS INDOMETHACIN-HYDROCHLOROTHIAZIDE IN THE TREATMENT OF NEPHROGENIC DIABETES INSIPIDUS.

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7.1 ABSTRACT

The effects of treatment with the combination amiloride-hydrochlorothiazide were compared to indomethacin-hydrochlorothiazide treatment, as yet considered to be the most effective treatment, in five boys with congenital nephrogenic diabetes insipidus. Amiloride-hydrochlorothiazide appeared to be equally effective as indomethacin-hydrochlorothiazide on urine volume and urine osmolality values. During the amiloride-hydrochlorothiazide period normokalemia could be maintained without potassium salt supplementation. Administration of amiloride-hydrochlorothiazide did not cause undesirable side effects. The combination may therefore be at least of similar value as the indomethacin-hydrochlorothiazide regimen in the treatment of this condition.

7.2 INTRODUCTION

Treatment of Nephrogenic Diabetes Insipidus (NDI) has posed a particularly vexing problem. Thiazide diuretics and prostaglandin synthesis inhibitors were shown to be of important benefit in the management of this disease. Several studies [1-2-3-4-5] have demonstrated that the combination of hydrochlorothiazide with the prostaglandin synthesis inhibitor indomethacin is the most effective therapy at this moment. However, prolonged use of thiazides is frequently complicated by hypokalemia, secondary to renal loss of potassium [6], and long-term treatment with prostaglandin synthesis inhibitors may have untoward renal and systemic side effects [7-8]. Therefore, we were interested in finding an alternative therapy with the same efficacy as the indomethacin-hydrochlorothiazide combination, but without the adverse effects. Recent work has suggested that the combination of hydrochlorothiazide with the potassium-sparing diuretic amiloride could be a satisfactory alternative [9]. In this latter study, however, only two patients with hereditary NDI were included, and other systematic studies comparing both forms of treatment are as yet not available.

The aim of the present study was to compare the effects of the indomethacin-hydrochlorothiazide combination with those of the amiloride-hydrochlorothiazide regimen in five children with NDI.

7.3 PATIENTS AND METHODS

The study was performed in five male patients (aged 5-16 years) with congenital NDI from the Netherlands. Two of them had a positive family history, the other three were isolated cases. Diagnosis of NDI was based on the typical history of polyuria, polydipsia, hypernatremia, fever, and failure to thrive in the presence of a highly diluted urine, and confirmed by the lack of increased urine osmolality after infusion of the synthetic vasopressin analogue 1-desamino-8-D-arginine vasopressin (DDAVP). In all patients serum creatinine and creatinine clearance were normal.
Echography revealed no hydronephrosis, hydroureter, or dilatation of the bladder. Acquired causes of NDI and additional tubular dysfunctions were ruled out in each patient.
All patients were receiving a diet with moderate salt restriction and treatment with indomethacin (2 mg/24 hour/kg/)-hydrochlorothiazide (2 mg/24 hour/kg), supplemented with oral potassium salt [1500 mg/24 hour]).
The studies lasted 16 days each and were performed at the pediatric metabolic unit of the St.Radboud Hospital of Nijmegen, The Netherlands. Informed consent was obtained from the parents and, when applicable, from the patients.
All patients were studied in the same way. A constant diet with moderate salt restriction, patterned after the home diet, was provided and all patients had free access to water. All intakes were recorded.
The study was divided into three consecutive periods:

Step I Continuation of the treatment with indomethacin (2 mg/24 hour/kg) - hydrochlorothiazide (2 mg/24 hour/kg, supplemented with oral potassium salt [1500 mg/24 hour]) for 6 days.

Step II Interval of 4 days in which only hydrochlorothiazide (2 mg/24 hour/kg, supplemented with oral potassium salt [1500 mg/24 hour]) was administered.

Step III Treatment with amiloride (20 mg/24 hour/1.73m^2)-hydrochlorothiazide (2 mg/24 hour/kg) for 6 days.

Each day, blood pressure and body weight were recorded by the medical staff. 24-hour urine was collected daily for measurement of volume, osmolality and creatinine. Blood was obtained by venipuncture on days 4, 5, and 6 of steps I and III, for measurement of sodium, potassium, creatinine and osmolality. Osmolar clearance (C_{osm}) and free water clearance (C_{H2O}) were calculated by conventional formulas:
$C_{osm} = (U_{osm} \times V) / P_{osm}$; $C_{H2O} = V - C_{osm}$, where U_{osm} represents urine osmolality, P_{osm} plasma osmolality, and V urine volume.
Data were analyzed for significance by Student paired t-test. The significance level was taken at 0.01.
After the study, the patients were discharged on the combined amiloride-hydrochlorothiazide regimen and moderate-salt diet and were re-evaluated weekly for the first month and four times a year thereafter.

7.4 RESULTS

Fig.1 presents the data collected in patient no.1.
The mean values of body weight, serum electrolytes, serum osmolality, glomerular filtration rate (GFR), urine volume, urine osmolality, osmolar clearance and free water clearance on days 4, 5, and 6 during treatment periods I and III are given in Tables 1 and 2 for each of the five boys. Serum sodium levels were significantly lower during the amiloride-hydrochlorothiazide period than during the indomethacin-hydrochlorothiazide period. No significant differences between the two forms of therapy were found with respect to the values of serum potassium, although in four patients

Table 7.1 Comparison of the two forms of treatment on body weight, serum electrolytes, serum osmolality and creatinine clearance in five patients with congenital nephrogenic diabetes insipidus. For each patient the mean values on day 4,5, and 6 are calculated.

			serum			
Patient		body weight (kg)	sodium (mmol/l)	potassium (mmol/l)	osmolality (mosmol/kg)	GFR (ml/min)
1	I	20.2	141	4.0	293	132
	A	19.6	136	3.3	287	116
2	I	24.5	146	3.7	292	125
	A	25.0	144	4.0	287	117
3	I	36.1	143	4.0	295	88
	A	36.2	137	4.4	280	96
4	I	49.5	145	3.8	295	89
	A	47.7	135	3.9	275	95
5	I	17.4	142	3.4	289	126
	A	16.8	138	4.7	291	103
p-value		n.s.	< 0.01	n.s.	n.s.	n.s.

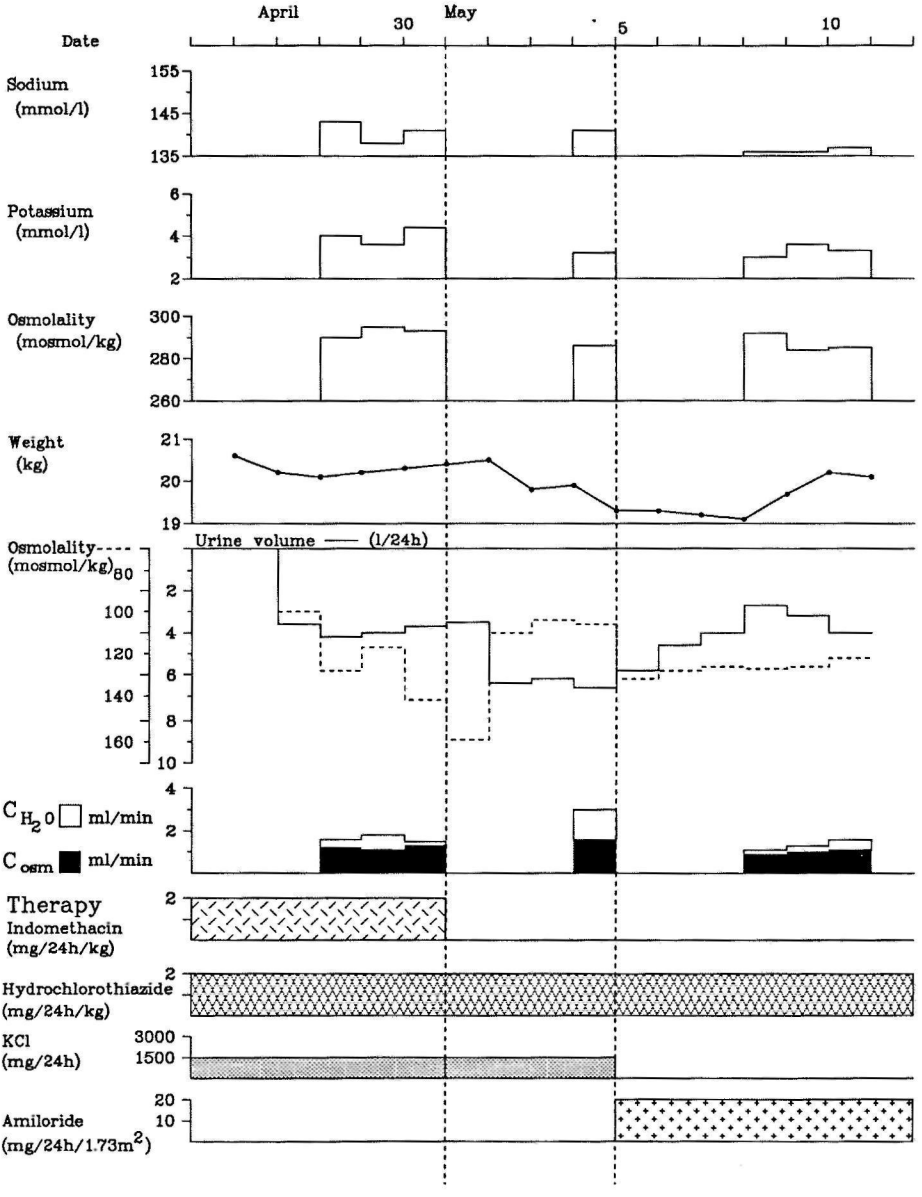
I = Indomethacin-Hydrochlorothiazide; A = Amiloride-Hydrochlorothiazide.

Table 7.2 Comparison of the two forms of treatment on urine volume, urine osmolality, osmolar clearance (C_{osm}), and free water clearance (C_{H2O}) in five patients with congenital nephrogenic diabetes insipidus. For each patient the mean values on day 4,5, and 6 are calculated.

Patient		volume (L/24h)	osmolality (mosmol/kg)	C_{osm} (ml/min)	C_{H2O} (ml/min)
1	I	4.0	129	1.2	1.6
	A	3.3	125	1.0	1.3
2	I	5.2	113	1.4	2.2
	A	5.2	126	1.6	2.0
3	I	2.9	282	1.9	0.1
	A	4.1	227	2.3	0.6
4	I	4.5	119	1.3	1.8
	A	5.3	137	1.9	1.8
5	I	2.5	139	0.8	0.9
	A	2.0	125	0.6	0.8
p-value		n.s.	n.s.	n.s.	n.s.

I= Indomethacin-Hydrochlorothiazide; A = Amiloride-Hydrochlorothiazide.

Fig. 7.1 Illustration of the effects of different forms of treatment in patient no.1.



potassium levels were slightly higher during the amiloride-hydrochlorothiazide period. Further comparison between the alternative treatments showed that there were no significant differences in the patients' body weight, and in serum osmolality, GFR, urine volume, urine osmolality, osmolar clearance and free water clearance.
Follow-up studies of up to 1 year revealed that the patients maintained normal serum electrolyte concentrations. Urine volume and osmolality remained within the same range as observed during the study. No side effects of the amiloride-hydrochlorothiazide combination were observed in four of our patients. In one patient amiloride administration had to be interrupted after 6 months because of abdominal pain and anorexia.

7.5 DISCUSSION

The treatment of NDI has been a problem since the original description of the disease in 1945 [10-11]. The first class of drugs shown to be relatively safe and clinically useful was the thiazide diuretics [12]. When combined with a reduction of solute (salt) intake, this mode of treatment reduces urine volume by only 20% to 50% of baseline values, and is therefore not entirely satisfactory. In addition, the thiazide-induced kaliuresis may cause further impairment of urine concentrating ability [13], which could exaggerate polyuria and polydipsia in patients with NDI. Another possible risk, associated with hypokalemia is cardiac arrhythmia [14]. Thus, simultaneous administration of potassium salt is necessary in most cases. Potassium supplements may cause severe gastrointestinal complications such as ulceration, gastritis, hemorrhage, obstruction and perforation [15-16-17] and, therefore, close supervision is necessary.
Prostaglandin synthesis inhibitors, such as indomethacin [1-2-3-4-5], ibuprofen [18], and acetylsalicylic acid [19] have been used for the treatment of NDI with encouraging results. Although the exact underlying mechanisms are not known, these agents appeared to have a beneficial effect on urinary output, especially when combined with

hydrochlorothiazide [2-3-4-5]. However, prolonged use of prostaglandin synthesis inhibitors is frequently complicated by severe side effects in the gastrointestinal, haematopoietic, and CNS-systems [7-8]. Gastrointestinal complaints and complications consist of anorexia, nausea, vomiting, abdominal pain, ulceration, perforation, and hemorrhage. Haematopoietic reactions include neutropenia, thrombocytopenia, and, rarely, aplastic anemia. In addition, renal dysfunction has been observed during indomethacin therapy, most often a transitory reduction in glomerular filtration rate (GFR) [2-3-4]. Thus, although the high efficacy of the indomethacin-hydrochlorothiazide regimen is without doubt, long-term treatment with this combination has major disadvantages.

Alon and Chan [9] demonstrated in two NDI patients that hydrochlorothiazide, when combined with the potassium-sparing diuretic amiloride, was at least as effective as the hydrochlorothiazide-prostaglandin synthesis inhibitor regimen. A systematic comparison between these two alternative combinations, however, was only made in one patient.

In the present study the observations by Alon and Chan are extended in five NDI patients. Urine volume- and urine osmolality values were not significantly different during either regimen, and no differences were seen with regard to their effects on osmolar clearance and free water clearance. Serum sodium levels were significantly lower with the amiloride-hydrochlorothiazide combination. Since the patients' weight and GFR were similar with both treatment modalities, this indicates that urinary sodium excretion was at a slightly higher level during the amiloride-hydrochlorothiazide period than during the indomethacin-hydrochlorothiazide trial. This is in agreement with previous studies which have shown that amiloride has natriuretic potency [20-21], whereas indomethacin may promote salt retention [22-23].

The use of amiloride in combination with hydrochlorothiazide in the treatment of NDI has three major advantages, which will be discussed in the following.

First, amiloride counterbalances the potassium losses secondary to thiazide treatment [24-25-26-27] and thus prevents hypokalemia and

the need for potassium salt supplementation. The potassium-sparing effect of amiloride was confirmed in our five patients with NDI. During the amiloride-hydrochlorothiazide period, there was no need for potassium supplementation to maintain normokalemia, in contrast to the indomethacin-hydrochlorothiazide regimen.

Second, the antidiuretic actions of thiazides and amiloride appear to be additive [9-25]. Different mechanisms of action within different segments of the distal nephron may provide a basis for the additive effects. As demonstrated in microperfusion studies [28-29-30] and microcatheterization experiments [31], thiazides inhibit the electroneutral sodium-dependent chloride transport in the "early" distal tubule, whereas amiloride blocks the luminal membrane sodium channel in the cortical and medullary collecting duct. Thus, combined administration of amiloride and hydrochlorothiazide results in a more marked urinary sodium excretion than either diuretic given as a single agent.

Assuming that the efficacy of thiazides in NDI is related to the reduction of extracellular sodium content and, therefore, enhancement of reabsorption in the proximal tubule, as proposed by Early and Orloff [32] and supported by others [33-34], further augmentation of sodium excretion by amiloride might be responsible for the additive antidiuretic effects of both drugs.

However, amiloride and hydrochlorothiazide might exert their effects in NDI also through other mechanisms. In this regard it is interesting to note that in vasopressin-deficient rats the antidiuretic effect of hydrochlorothiazide was shown to be mediated not only by changes in proximal tubular function, but, more importantly, by a rise in inner medullary osmolality, favouring increased water reabsorption form the collecting duct [35]. Likewise, the antidiuretic effect of amiloride has been shown to be, at least in part, independent of increased proximal reabsorption in patients with lithium-induced NDI. [36]. Whatever the explanation, the fact that the antidiuretic actions of amiloride and hydrochlorothiazide are additive is important in the treatment of NDI. Simultaneous administration of these drugs significantly attenuates obligatory urine loss to a tolerable level per day. Such a change in urine

flow also prevents the dilatation of the urinary tract.
Finally, side effects of amiloride are uncommon and less severe than those reported for indomethacin, and several studies, including the present one, have indicated that prolonged use of the drug in combination with hydrochlorothiazide is usually well tolerated [9-37-38].

We conclude that the amiloride-hydrochlorothiazide combination is a desirable form of therapy for congenital nephrogenic diabetes insipidus. It is equally effective as the prostaglandin synthesis inhibitor-hydrochlorothiazide regimen, it prevents the need for potassium supplementation, and it has only minor long-term side effects.

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CHAPTER 8

GENERAL DISCUSSION

If not diagnosed and treated early in life, NDI may have serious complications such as growth retardation and brain damage, the latter resulting in severe mental retardation. Furthermore, the disease may have a serious impact on the social life of families in which one or more children are suffering from NDI.
The study described in this thesis has been undertaken in order [i] to search for reliable means of early diagnosis and carrier detection in X-linked NDI, [ii] to extend our knowledge about the pathogenesis of the disorder, and finally [iii] to find an alternative for the conventional therapy (the combination indomethacin-hydrochlorothiazide) of the disease, the latter having major disadvantages.
The following discussion will be focused on the aspects of diagnosis, pathogenesis and therapy of NDI. Finally, an outlook on further studies will be given.

8.1 DIAGNOSIS

Employing genetic DNA linkage studies in 11 families with NDI, we were able to demonstrate tight linkage with the anonymous DNA markers DXS52, DXS33, DXS15, DXS134, the clotting factor VIII and the colour blindness genes, all of which are located at band Xq28 on the distal long arm of the human X chromosome (chapter 2, 3, and 4). The highest lod score (log of odds for linkage versus independent assortment given the observed segregation in families) of 10.35 was obtained with the DXS52 marker, defined by probe St14, which did not show recombination with the disease gene. This indicates that the odds were 10^{10}:1 in favor of linkage. From these results it can be concluded that the distance between the NDI gene and the locus DXS52 is less than 5 centiMorgans (cM) (95% confidence limits). This renders the DXS52 marker a valuable tool for heterozygote detection and early (prenatal) diagnosis of NDI. In addition, multipoint linkage analysis enabled us to construct a refined genetic map of the distal part of the long arm of the human

X chromosome : Xcen - F9- DXS98 - F8C/CBD,CBP - NDI/DXS52 - DXS134-Xqter. This map places the NDI gene between the coagulation factor VIII gene (F8C) and DXS134. It is worth pointing out that, although our analysis maps the F8C gene proximal to the DXS52 locus, their is still no consensus on the relative orientation of both genes. During the most recent Human Gene Mapping Conference (HGM10, Mandel et al., 1989) both studies suggesting that F8C is proximal to DXS52 (Lehesjoki et al., 1989), and studies suggesting a distal localization of F8C with respect to DXS52 (Gross et al., 1989; Vincent et al., 1989) have been reported.
The refined localization of the NDI gene achieved in our study should be a major step towards the isolation of this gene by application of 'reverse genetics' strategies (Ruddle, 1984; Orkin, 1986), which will be the purpose of further studies (see 'Outlook: Towards isolation of the NDI gene').

8.2 PATHOGENESIS

It has been demonstrated that patients with X-linked NDI fail to show elevated von Willebrand factor (vWF)-, FVIII coagulant activity (FVIII:C)- and plasminogenactivator levels after administration of the V_2 specific agonist 1-desamino-8-D-arginine vasopressin (DDAVP) (this study: chapter 5 and 6; Kobrinski et al., 1985; Bichet et al., 1988). In addition, vasodilatory responses to DDAVP are absent in NDI patients (this study: chapter 5 and 6; Bichet et al., 1988). These findings suggest that the hemodynamic-, coagulation-, and fibrinolytic responses to DDAVP are mediated by activation of extrarenal V_2 receptors and support the concept of a general V_2 abnormality in NDI. The precise location of the extrarenal V_2 receptors remains to be elucidated. Possible sites are the endothelium [the site of synthesis and secretion of plasminogen-activator (Gerard et al., 1986) and of vWF (Jaffe et al., 1974)], the hepatic sinusoids [the site of FVIII synthesis and release (Stel et al.,1983)] and the central nervous system (Cash et al., 1978).

It is worth noting that a few patients have been reported, among whom the patient (J.B.) described in chapter 6, in which the defect seems to be confined to the kidney (Brenner et al., 1988; Moses et al., 1988). These patients, however, most probably do not suffer from 'classical' NDI but from a variant type of the disease. The following discussion will be focused on the 'classical' type of NDI. Subsequently, some comments on the variant type of the disease will be made.

As yet, the precise pathogenetic mechanism underlying NDI is undefined. Nevertheless, the results of DDAVP experiments strongly argue against several of the potential defects dealt with in the 'Introduction' (1.2.7), as will be discussed below. Moreover, other hypothetical sites of the primary defect may be excluded by drawing an analogy with pseudohypoparathyreoidism. This disorder results from primary resistance to the action of parathormone (PTH) which, like vasopressin, acts on the renal tubule by binding to the hormone receptor and subsequently through activation of the adenylate cyclase-cAMP system. As to the defects proximal to cAMP formation, deficient activity of the guanine nucleotide binding protein (G protein) is not very likely. The G-protein involved in adenylate cyclase activation is assumed to be similar if not identical in most cells (Lochrie and Simon, 1988) (although tissue-specificity of G-proteins can not be excluded completely [see chapter 6]). Therefore, a defect in this G-protein should lead to resistance to multiple hormones that act by stimulating adenylate cyclase. Indeed, deficiency of this G-protein has been shown to be the basis for multiple hormone resistance in patients with pseudohypoparathyreoidism type I (Spiegel et al., 1982; Levine et al., 1983). A defect of the enzyme adenylate cyclase per se, such as has been found in a subset of patients with pseudohypoparathyreoidism (Barret et al., 1989), can also be excluded by the fact that multiple hormone resistance is not observed in NDI. For the same reason it is improbable that an increase in cAMP phosphodiesterase, resulting in abnormally high degradation of cAMP, is underlying NDI.

As to the defects distal to cAMP formation, an abnormality of the cAMP-dependent protein kinase can be considered a candidate for

causing NDI. The enzyme cAMP dependent protein kinase exists as two types (types I and II), which are distinguished by their different regulatory subunits (RI and RII, respectively) whereas the catalytic subunit (C) of both types appear essentially identical (Flockhart and Corbin, 1982; Krebs, 1989). It has been demonstrated that several unique and antigenetically distinct gene products exist within each R-subunit class. Some appear to be expressed in most tissues (Lee et al., 1983), while expression of others is tissue-specific (Jahnsen et al., 1986). An attractive speculation might be that an abnormality in a R-subunit, which is specific for the tubular cells sensitive to vasopressin, is the cause of NDI. This assumption, however, does not explain the absence of extrarenal responses to vasopressin in NDI patients.

In the animal model of NDI lack of insertion of water permeable patches into the apical membrane of vasopressin sensitive renal cells has been demonstrated (Brown et al., 1985). The observation that the resistance to vasopressin in patients with X-linked NDI is not confined to the kidney but involves other, extrarenal, tissues as well, renders it improbable that the defect in human NDI is identical to that found in the experimental disease.

In a recent study, Bichet et al. (1989) infused epinephrine into three male subjects with NDI. Factor VIII and tissue type plasminogen activities increased by 75 to 100 %, and plasma renin activity and plasma cyclic AMP by 200 %. None of these values changed when patients received DDAVP. These data strongly argue for an altered pre-cAMP mechanism.

As yet, the most likely explanation for the resistance to vasopressin in NDI is absence or abnormality of the V_2 receptor itself. In view of the specificity of the different hormone receptors, this hypothesis is consistent with the fact that NDI is characterized by insensitivity to a single specific hormone. Moreover, the idea is compatible with the fact that both renal- and extrarenal V_2-mediated responses are absent in this disease.

Whether or not the renal and extrarenal V_2 receptors are exactly identical remains to be elucidated. In this respect, the finding of normal V_2-mediated responses to DDAVP in a few 'atypical' NDI

patients is very interesting. It is tempting to speculate that these patients have a V_2 receptor defect which is limited to the kidney and does not include the extrarenal receptors. This assumption can only be valid if the extrarenal receptor is different from the renal one. As mentioned in the 'Introduction' (1.2.4), the mammalian renal V_2 receptor consists of two subunits (Fahrenholz et al., 1985). Therefore, it is conceivable that the renal and extrarenal receptors share one subunit but have different second subunits. Thus, the 'classical' type of NDI might be due to a defect of the common subunit, resulting in a generalized V_2 receptor defect. In the patients with the variant type of NDI, there might be lack or a functional defect of the second subunit of the renal receptor, resulting in a V_2 receptor defect that is confined to the kidney.
Alternatively, the 'atypical' patients may have a defect distal to the formation of cAMP. In patient J.B. (chapter 6), this was considered unlikely (but not excluded), given the lacking rise of urinary cAMP levels in response to DDAVP. In the few other 'atypical' patients reported (Moses et al., 1988; Brenner et al., 1988), urinary cAMP levels were not measured.

8.3 THERAPY

In recent years it has been shown that prostaglandin synthesis inhibitors, such as indomethacin, are very useful in the treatment of NDI, especially when combined with hydrochlorothiazide (Blachar et al., 1980; Monnens et al., 1984; Rasher et al.,1987). The serious disadvantages inherent to the use of the prostaglandin synthesis inhibitor-hydrochlorothiazide regimen, however, led us to look for another approach. Based on a recent study, in which the value of the combination amiloride-hydrochlorothiazide in the treatment of NDI was suggested (Alon and Chan, 1985), we systematically compared the effects of the amiloride-hydrochlorothiazide regimen with those of the indomethacin-hydrochlorothiazide combination. We demonstrated that both combinations were equally effi-

cient in decreasing urine volume and increasing urine osmolality (chapter 7). Use of the combination amiloride-hydrochlorothiazide has several important advantages. First, amiloride counterbalances the potassium losses secondary to prolonged use of thiazides (Alon and Chan, 1985; Dyckner et al., 1988) and thus prevents hypokalemia. Secondly, the antidiuretic actions of amiloride and hydrochlorothiazide appear to be additive (Alon and Chan, 1985; Wilson et al., 1988). Different sites of action of both drugs within the distal nephron (Fig. 1) may underlie their additive effects (Costanzo, 1985; Velazquez and Wright, 1986; Ellison et al., 1987; Sonnenberg et al., 1987).

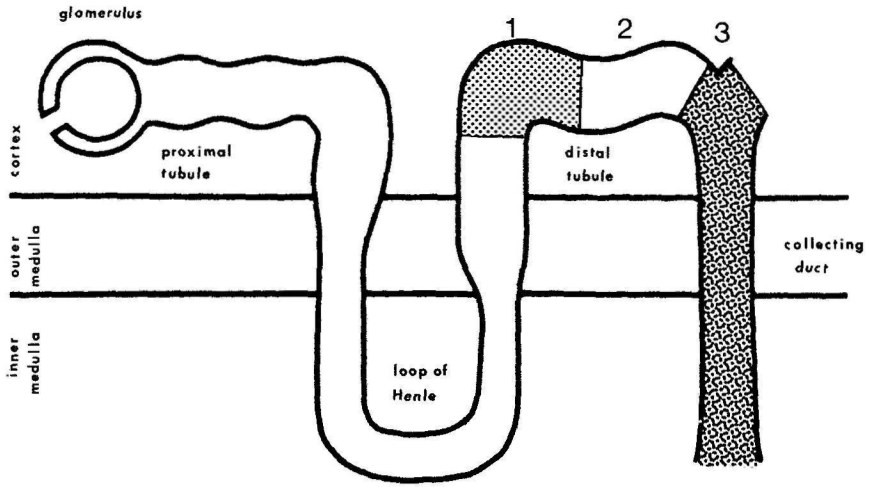

Fig. 8.1

Schematic presentation of the different sites of action of thiazides and amiloride in the distal nephron. The distal tubule is heterogeneous and comprises at least three succesive segments: the distal convoluted tubule (1), the connecting tubule (2), and the initial collecting tubule (3). Thiazides act on the distal convoluted tubule (the 'early' distal tubule), while amiloride acts on the initial collecting tububle (the 'late' distal tubule) and on the cortical and medullary collecting duct.

Finally, several studies, including the present one, have indicated that the amiloride-hydrochlorothiazide regimen has only minor long term side effects.
Taken together, the combination amiloride-hydrochlorothiazide may be a satisfactory alternative to the prostaglandin synthesis inhibitor-hydrochorothiazide regimen in the treatment of NDI.

8.4 OUTLOOK: TOWARDS ISOLATION OF THE NDI GENE

For most of the genes that have been cloned to date, isolation was possible because of their known metabolic function or because the purified gene product was available. However, most human single gene disorders, including NDI, result from mutations in genes whose normal function is unknown. In the absence of a characteristic metabolic disorder or a specific functional test, the most promising approach to elucidating such gene defects is the use of 'reverse genetics' strategies (Ruddle, 1984; Orkin, 1986; Ropers, 1987). In this approach the isolation of the respective disease gene relies solely on its chromosomal location. Reverse genetics strategies have been applied succesfully for cloning the Duchenne Muscular dystrophy (DMD) gene (Monaco et al., 1986; Burghes et al., 1987; Koenig et al., 1987), the chronic granulomatous disease (CGD) gene (Royer-Pokora et al., 1986), the retinoblastoma (Rb) gene (Friend et al., 1986; Lee et al., 1987) and, most recently, for isolation of the gene responsible for cystic fibrosis (Riordan et al., 1989; Rommens et al., 1989).
The detailed information on the subchromosomal localization of the NDI gene obtained in our study makes it potentially feasible to approach the isolation of the gene itself in a similar fashion. Alternatively, in view of the evidence that a defect in the V_2 receptor may be the primary cause of NDI, cloning strategies might involve functional assays for presence of this receptor. In the following section both approaches will be discussed in some detail.

Reverse genetics approach

The first crucial step to identify and clone genes using the reverse genetics approach is to establish the exact map position of the gene involved. Our linkage analyses have placed the NDI gene in the Xq28 region within 5 cM from the most closely linked marker DXS52 (Knoers et al., 1988a [chapter 2], 1988b [chapter 3], 1989 [chapter 4]). The genetic length of the entire human genome, about 3000 cM, corresponds to a physical length of around 3×10^9 bp. Although cross-overs are not evenly distributed in the human genome (Saadallah and Hulten, 1983), 5 cM should on average represent about 5×10^6 bp, which is an enormous distance in molecular genetic terms. Moreover, it is not yet known whether the NDI gene is located proximal or distal to the DXS52 locus.

Therefore, further definition of the chromosomal region carrying the NDI gene will require additional linkage studies, using new DNA markers from the Xq28-qter region, which have recently become available. The number of available NDI families will ultimately limit the precision with which genetic distances and the gene order in the NDI region can be determined. In general, linkage analysis alone will get us not much closer than 1 cM from the disease gene. Even a physical distance of 10^6 bp (which corresponds, on average, to a genetic distance of 1 cM) is too large to be bridged by standard molecular techniques.

The length of region easily analyzed by conventional techniques has until recently been limited by the capacity of available cloning vectors (<40 kb). In addition, the resolution of conventional gel electrophoretic methods is only adequate for fragments of about 20.000 base pairs or smaller. To bridge the gap between genetic and physical mapping approaches the techniques of pulsed field gel electrophoresis (PFGE) (Schwartz and Cantor, 1984; Carle and Olson, 1984; van Ommen and Verkerk, 1986), field inversion gel electrophoresis (FIGE) (Carle et al., 1986) or contour-clamped homogeneous electric field (CHEF) electrophoresis (Chu et al., 1986) can be employed. These methods are capable of resolving DNA fragments up to ten million base pairs by periodicallly altering the direction of the electric field. Used together with methods for cutting DNA

into large fragments (van Ommen and Verkerk, 1986), these techniques allow the construction of a long range physical map that encompasses the gene of interest. Moreover, PFGE is ideally suited for the detection of disease-associated microdeletions. Submicroscopic deletions have been found in a number of X-linked disorders (Wieringa et al., 1985; Gal et al., 1986; Cremers et al., 1987) and have been a great asset for the molecular and genetic characterization of several genes (Monaco et al., 1986; Royer-Pokora et al., 1986; van Ommen et al., 1986; Page et al., 1987).

With the identification of very closely linked markers or microdeletions it should be possible to clone the entire region containing the disease gene by employing 'chromosome walking' and 'jumping' techniques.

'Chromosome walking' (Bender et al., 1983) consists of the iterative screening of genomic (phage or cosmid) libraries to isolate, in sequential steps, overlapping DNA clones that together span up to several 100 kb of chromosomal DNA. At present, the capacity of the available cloning vectors (phages or cosmids) limits the distances that can be analyzed by walking techniques. On average, the new cosmid clones will only contain 20 kb of additional DNA and the maximum distance covered per step will be less than 40 kb. Therefore, this approach is essentially unsuitable for covering large distances. Moreover, the method has no directionality: there is an equal chance of walking away from or towards the NDI gene.

In order to move along chromosomes rapidly, an elegant strategy has been developed called 'chromosome jumping' or 'hopping' (Collins and Weissman, 1984). In this approach distances of 50-500 kb can be covered in a single cloning step. This is achieved by first ligating very large genomic restriction fragments into covalent DNA circles containing a selectable marker at the junction site. Thereafter, the DNA circles are digested with a restriction enzyme that does not cleave the marker sequence, and the resulting fragments, carrying both ends of the original long genomic DNA sequence, as well as the selectable marker, are cloned into conventional phage vectors. Using this type of protocol various libraries have been constructed (Poustka and Lehrach, 1986; Collins et

al., 1987; Poustka et al., 1987) which allow hopping along a chromosome to move rapidly from a linked marker to a disease gene. Concepts have been devised to pre-determine the direction of chromosome hopping.

A recent, promising approach which dramatically extends the limit of contiguously clonable DNA (from 40 kb to 500-1000 kb or more) is the development of yeast artificial chromosome (YAC) cloning (Burke et al., 1987). This will simultaneously deviate the problems of 'chromosome walking' (tedious and sequential) and 'hopping' (loss of intermediate DNA). In combination with the reduced complexity cell hybrid techniques (see below), it will make the 'wholesale' cloning of a target region conceivable.

In addition to molecular genetic techniques, the use of somatic cell techniques (Ruddle et al., 1981) may help to further define and isolate the chromosome segment that carries the NDI gene. Somatic cell hybrid cell lines containing various portions of the telomeric part of the X chromosome long arm will help to order DNA markers in the region of interest. For this purpose, we can make use of a human-hamster hybrid cell line (908K1B17), which contains -as the only human material- the centromeric region of chromosome 19 and the distal long arm of the X chromosome (Schonk et al., 1989). This cell line has been constructed by irradiation-induced breakage (Goss and Harris, 1975) of a parental cell hybrid (908K1) carrying a X/19 translocation. Pilot studies have already shown that this cell line carries all DNA markers that are tightly linked to NDI and has lost the more proximal markers which are only loosely linked to NDI.

Further fragmentation of the distal part of Xq into separable subsegments is possible through improved irradiation-based protocols (Benham et al., 1989). Where these subsegments include the NDI region, a complete molecular analysis will be possible.

With the somatic cell genetic and the molecular approaches described above, it should be possible to isolate the entire region in which the NDI locus resides, but without specific biological tests identification of the recombinant DNA clone carrying the relevant genetic information is still very tedious. In genomic DNA,

the compound of non-coding sequence significantly exceeds that of coding sequence (the exons). To identify expressed sequences in a collection of cloned probes, at least two approaches are possible. The first one is based on the expectation that exons are more stringently conserved during evolution than non-coding sequences. Therefore, exons can be isolated by identifying non-repetitive DNA segments within the cloned region that detect homologous sequences in DNA from other mammalian or vertebrate species. In this way, candidate genes for portions of the DMD gene (Monaco et al., 1986), for the CGD gene (Royer-Pokora et al., 1986), for the Rb gene (Friend et al., 1986), and very recently for the cystic fibrosis gene (Rommens et al., 1989) have been isolated. A second strategy to detect expressed sequences is based on the knowledge that many vertebrate genes are preceeded by DNA sequences that are rich in the non-methylated CG dinucleotides (Bird et al., 1986). These sequences, known as 'HTF islands' (HpaII tiny fragment), have a length of between 500 and 2000 bp, and often include the first exons as well as upstream sequences 5' to the associated gene. GC-rich 'HTF islands' can be easily detected because, in contrast to most other human DNA sequences, they will be cleaved by many rare cutting restriction enzymes that contain CG dimers in their recognition sequence. This approach has been employed in an attempt to identify the gene responsible for cystic fibrosis (Estivill et al., 1987).

Once a candidate gene has been isolated, definite proof for its involvement in the etiology of NDI must then come from studies demonstrating lack or aberrant expression of this gene in NDI patients.

Rapid analysis of mutant genes is now possible using the new Polymerase Chain Reaction (PCR) technique (Saiki et al., 1985), a procedure in which specific DNA segments are amplified enzymatically in vitro by DNA polymerase, making use of short synthetic DNA primers that flank the region of interest. 20 to 30 PCR cycles allow to amplify the number of relevant DNA sequences several million-fold in a few hours, thereby enabling their direct molecular (and sequence) analysis.

Thus, with the PCR procedure it is possible to rapidly amplify the

relevant genes from genomic DNA of patients and to screen this DNA for deletions or point mutations.

The "functional approach"

Conventional methods for cloning the V_2 receptor require purification of the receptor and identification of the amino-acid sequence, either in whole or in part, of the receptor protein(s). Then, oligonucleotides corresponding to short stretches of the amino acid sequence can be used as probes to screen cDNA or genomic libraries for recombinant clones that contain homologous sequences. These oligonucleotides can also be used as potential primers for the synthesis of highly enriched cDNA. Alternatively, if antibodies specifically directed to the receptor protein(s) were available, these could be used as probes to screen expression libraries for suitable recombinant clones (Young and Davis, 1983). Unfortunately, however, neither of these approaches is feasible for the cloning of the NDI gene because the structure of the V_2 receptor remains to be elucidated and suitable antibodies are not available yet.

Very recently, an interesting observation was made, which may provide the basis for an alternative cloning strategy. Using a functional assay, based on the increase of intracellular cAMP after addition of vasopressin in the medium, V_2 receptor activity was measured in the 908K1B17 somatic cell hybrid line (see earlier), which contains the distal part of the long arm of the human X chromosome, but not in Chinese hamster parental cells which served as controls (Dr. F. Fahrenholz, personal communication). On the basis of this observation, it should be possible to devise cloning and transfection protocols to enrich for and isolate genomic sequences that encompass the NDI gene. If succesful, further functional proof for the identity of the genes isolated in this way could then come from complementation assays, using a cell line deficient in vasopressin binding (for instance, the mutant pig kidney cell line isolated by Fahrenholz and co-workers [Jans et al., 1986]). Experiments along this line are in progress.

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The studies reported in this thesis were undertaken with the aim of finding reliable means of early diagnosis and carrier detection of NDI and in order to increase our understanding of the pathogenesis of this disorder. In addition, in view of the disadvantages of the conventional therapy of NDI, an alternative regimen was tested for its efficiency and safety.

The 'Introduction' (chapter 1) summarizes the literature available on the clinical picture of NDI, its history, definition and clinical manifestation, diagnosis, genetics, and treatment. Subsequently, as a prerequisite for a discussion of the potential pathophysiological mechanism(s) involved in NDI, an extensive review of the literature on the normal water handling is presented. Attention is paid to the antidiuretic hormone (vasopressin) release, the generation and maintenance of an osmotic gradient in the medullary interstitium, and the modulation of water and solute transport by vasopressin.
A brief survey of the extrarenal effects of vasopressin and a description of an animal model for NDI have been included.
Thereafter, possible pathophysiological mechanisms that could underly the renal unresponsiveness to vasopressin in NDI are offered.

The results of DNA linkage studies with various X-linked restriction fragment length polymorphisms (RFLPs) are presented in the chapters 2, 3, and 4. By performing two-point linkage analysis in NDI families (chapter 2 and 3) we were able to demonstrate tight linkage between the NDI gene and the DNA marker loci DXS52, DXS15, F8C, and DXS134, all of which are located within band Xq28. These results have assigned the NDI gene to the subtelomeric region of the X chromosome long arm. By employing three-point linkage analysis with six DNA markers from the Xq28 region (chapter 4) we were able to define the position of the NDI locus more precisely and to construct a genetic map of the Xq28 region.

The closely linked DNA markers, that have been identified in this study provide a valuable tool for reliable carrier detection and early (prenatal) diagnosis. In addition, the refined genetic mapping of the NDI locus may pave the way towards isolating the gene itself.

The hemodynamic, coagulation, and fibrinolytic responses to administration of the specific V_2 agonist 1-desamino-8-D-arginine vasopressin (DDAVP) in NDI patients and normal controls are presented in the chapters 5 and 6. In normal controls, significant increases in vWF:ag- and plasminogenactivator levels, and considerable vasodilatory responses were observed directly after DDAVP infusion. None of these changes were seen in NDI patients, with the exception of one patient (J.B.) who exhibited completely normal responses (chapter 6). The results confirm that the DDAVP-induced changes in hemodynamic, coagulation, and fibrinolytic parameters depend on extrarenal V_2 receptor activation and support the concept of a general V_2 receptor defect in NDI. It is suggested that patient J.B. may have a variant type of the disease, in which the V_2 abnormality is confined to the kidney.
In addition, fibrinolytic parameters were examined in female NDI carriers (chapter 5). Responses were rather variable. It is concluded that DDAVP tests are of limited use for the detection of NDI carriers.

The combination of indomethacin-hydrochlorothiazide is, as yet, the most effective form of therapy. Prolonged use of this combination, however, has several important disadvantages. In chapter 7, studies are presented that compare the effects of the indomethacin-hydrochlorothiazide therapy with those of an alternative regimen, the combination of amiloride-hydrochlorothiazide. Both combinations appeared to be equally effective in reducing urine volume and increasing urine osmolality. Because of several advantageous aspects of amiloride-hydrochlorothiazide therapy, we conclude that this regimen can be a satisfactory alternative to the indomethacin-hydrochlorothiazide combination in the treatment of NDI.

In the general discussion (chapter 8) the importance of the localization of the NDI gene for diagnostic purposes is emphasized. As to the pathogenesis of NDI, it is concluded that absence or defective functioning of the V_2 receptor forms the most likely explanation for the renal (and extrarenal) unresponsiveness to vasopressin.
Finally, an outlook is given on the isolation of the NDI gene, which should lead to elucidation of the biochemical defect underlying NDI.

Dit proefschrift is gewijd aan een aantal aspecten van de congenitale, geslachtsgebonden nierziekte Nefrogene Diabetes Insipidus (NDI). De studie had ten doel een betrouwbare methode voor vroegdiagnostiek te vinden en een beter inzicht te verkrijgen in de pathogenese van de stoornis.
Gezien de nadelen verbonden aan de huidige behandeling van NDI werd bovendien een alternatieve therapievorm op zijn efficiëntie en veiligheid getest.

In de Inleiding (hoofdstuk 1) wordt een literatuuroverzicht gegeven van de klinische aspecten van NDI. Geschiedenis, definitie en symptomatologie, diagnostiek, erfelijkheid, en behandeling worden achtereenvolgens besproken.
Vervolgens wordt aandacht besteed aan het normale concentratiemechanisme van de nier, omdat inzicht in dit proces noodzakelijk is voor de bespreking van de mogelijke pathofysiologische oorzaken van NDI. Behandeld worden de afscheiding van vasopressine uit de hypofyse, de opbouw en het behoud van een osmotische gradiënt in het niermerg, en de invloed van vasopressine op de water- en zouthuishouding van de nier. Een korte schets van de extrarenale effecten van vasopressine en een beschrijving van een diermodel van NDI zijn aan dit hoofdstuk toegevoegd.
Daarna worden de pathofysiologische mechanismen die ten grondslag zouden kunnen liggen aan de ongevoeligheid voor vasopressine in NDI vermeld.

De resultaten van DNA-koppelingsstudies met verschillende X-gebonden restrictie fragment lengte polymorphismen (RFLPs) worden gepresenteerd in de hoofdstukken 2, 3 en 4. Door middel van tweepunts koppelingsanalyses (hoofdstukken 2 en 3) werd nauwe koppeling aangetoond tussen het NDI-gen en de DNA merkgenen DXS52, DXS15, F8C, en DXS134, die alle in band Xq28 gelokaliseerd zijn. Op grond van deze bevindingen kon het NDI-gen worden toegewezen aan het subtelomere gebied van de lange arm van het X-chromosoom. Met

behulp van drie-punts koppelingsanalyses met 6 DNA merkgenen gelokaliseerd in de Xq28-regio (hoofdstuk 4) kon de positie van het NDI-gen nog nauwkeuriger worden bepaald en kon een genetische kaart van het Xq28-gebied worden geconstrueerd.
De nauw gekoppelde DNA merkgenen, die in deze studie geidentificeerd zijn, verschaffen ons waardevolle instrumenten voor de herkenning van draagsters en voor vroege (prenatale) diagnostiek. De nauwkeurige lokalisatie van het NDI-gen is een eerste stap op weg naar de isolatie van het gen zelf.

De hemodynamische-, stollings- en fibrinolytische effecten in NDI-patiënten en normale controlepersonen na toediening van de specifieke V_2 receptoragonist 1-desamino-8-D-arginine vasopressine (DDAVP) worden gepresenteerd in de hoofdstukken 5 en 6. Significante toename in vWF:ag- en plasminogeenactivatorspiegels en een duidelijke vasodilatoire reactie werden waargenomen in normale personen direct na DDAVP infusie. Bij NDI-patiënten bleven de hemodynamische-, stollings- en fibrinolytische parameters na toediening van DDAVP echter ongewijzigd. Een patiënt (J.B.) vormde daarop een uitzondering (hoofdstuk 6).
De resultaten bevestigen dat de door DDAVP geinduceerde veranderingen in hemodynamische-, stollings-, en fibrinolytsiche parameters worden gemedieerd door extrarenale V_2 receptoractivatie. De bevindingen zijn in overeenstemming met de hypothese dat de V_2 receptorafwijking in NDI gegeneraliseerd is. Er wordt gesuggereerd dat patiënt J.B. zou kunnen lijden aan een variant type van de ziekte[C], waarin de V_2 receptorafwijking beperkt is tot de renale tubuli.
Tevens werden de fibrinolytische parameters na DDAVP toediening gemeten in draagsters van het NDI-gen (hoofdstuk 5). De reacties waren buitengewoon variabel. Er wordt geconcludeerd dat DDAVP testen slechts van beperkte waarde zijn voor de opsporing van NDI-draagsters.

Op dit moment is de combinatie indomethacine-hydrochlorothiazide de

meest effectieve vorm van therapie voor NDI. Er zijn echter belangrijke nadelen verbonden aan langdurig gebruik van deze combinatie. In hoofdstuk 7 worden de effecten van de combinatie indomethacine-hydrochlorothiazide vergeleken met die van een alternatief regime, de combinatie amiloride-hydrochlorothiazide. De resultaten laten zien dat de combinatie amiloride-hydrochlorothiazide van even grote waarde kan zijn in de behandeling van NDI als de combinatie indomethacine-hydrochlorothiazide. De voordelen van gebruik van amiloride-hydrochlorothiazide worden beschreven.

In de einddiscussie (hoofdstuk 8) worden de bevindingen van deze studies besproken. Het belang van de lokalisatie van het NDI-gen voor diagnostische doeleinden wordt benadrukt. Wat de pathogenese van NDI betreft, wordt geconcludeerd dat afwezigheid of een afwijking van de V_2 receptor zelf de meest waarschijnlijke verklaring vormt voor de renale (en extrarenale) ongevoeligheid voor vasopressine in deze ziekte.
Tot slot wordt aangegeven welke wegen bewandeld kunnen worden om te geraken tot isolatie van het NDI-gen, en daarmee tot ontrafeling van het biochemische defect.

Op deze plaats wil ik iedereen bedanken die op enigerlei wijze heeft bijgedragen aan de totstandkoming van dit proefschrift. Enkele personen wil ik graag speciaal noemen.
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Bij de uitwerking van, en de discussies over de meerpunts-koppelings-analyses leverde Drs. H. Brunner (afdeling Klinische Genetica) een belangrijke bijdrage.
De statistische bewerking van de in de hoofdstukken 5 en 6 beschreven resultaten werd met kritische accuratesse verricht door Drs. A. Teeuwis (Mathematisch-Statistische Adviesafdeling).
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Paul, jouw steun was onontbeerlijk.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren te Nijmegen op 9 augustus 1958. In 1976 behaalde zij het diploma gymnasium β (Canisius College - Mater Dei, te Nijmegen). In 1977 begon zij haar studie in de Geneeskunde aan de Katholieke Universiteit te Nijmegen. In 1983 slaagde zij voor haar doctoraal examen, in januari 1986 werd het artsexamen afgelegd. Van november 1986 tot november 1989 was zij als wetenschappelijk medewerkster verbonden aan de afdelingen Kindergeneeskunde (subafdeling Kindernefrologie, onder leiding van Prof. Dr. L.A.H. Monnens) en Anthropogenetica (onder leiding van Prof. Dr. H.H. Ropers) van het St. Radboudziekenhuis te Nijmegen. In deze periode werd het hier beschreven promotieonderzoek uitgevoerd. Dit onderzoeksproject werd gesubsidieerd door de Nier Stichting Nederland.
Sinds december 1989 is zij in opleiding tot klinisch geneticus op de afdeling Humane Genetica van het St. Radboud Ziekenhuis te Nijmegen (opleider: Dr. B. Hamel).

De auteur is getrouwd met Paul G. van Slobbe sinds april 1989.

STELLINGEN behorend bij het proefschrift 'Nephrogenic Diabetes Insipidus' van V.V.A.M. Knoers.

I

De nauwkeurige lokalisatie van het gen voor Nefrogene Diabetes Insipidus in de Xq28-regio is een eerste belangrijke stap op weg naar de isolatie van dit gen.

Dit proefschrift

II

De renale (en extrarenale) ongevoeligheid voor arginine vasopressine in congenitale Nefrogene Diabetes Insipidus wordt hoogst waarschijnlijk veroorzaakt door een gegeneraliseerd V_2-receptor defect.

Dit proefschrift

III

Ten onrechte beweerden Block et al. dat de vasopressine V_2-receptor ook op mononucleaire fagocyten aanwezig is.

Block, L.H., et al., J. Clin.Invest. 68:374-381, 1981

Eigen waarneming

IV

De bindingskarakteristieken (maximaal aantal bindingsplaatsen [1] en affiniteit voor vasopressine [2]) van V_1-receptoren op bloedplaatjes in patiënten met Nefrogene Diabetes Insipidus (NDI) zijn identiek aan die van de plaatjesreceptoren in controlepersonen. Dit impliceert de aanwezigheid van intacte V_1-receptoren in NDI.

Eigen waarneming

V

Het verschijsel polydipsie mag pas dan als psychogeen worden beschouwd wanneer alle overige mogelijke oorzaken zijn uitgesloten.

VI

Bij kinderen met een bijnierinsufficiëntie dient steeds de diagnose adrenoleukodystrofie overwogen te worden.

O'Neill, B.P., et al. Neurol. 32:543-547, 1982

VII

Met behulp van de positron emissie tomografie (PET-scan) kan enig inzicht worden verkregen in het pathofysiologisch mechanisme dat ten grondslag ligt aan onbegrepen cerebrale verschijnselen zoals chorea.

Hosokawa, S., et al. J.Neurol.Neurosurg.Psych. 50:1284-1287, 1987

VIII

Het meest waarschijnlijke produkt van een 45,X/46,XY zwangerschap is een fenotypisch normale jongen.

Chang, H.J., et al. Am.J.Hum.Genet. 46:156-167, 1990

IX

De fenotypisch sterk verschillende Prader Willy en Angelman syndromen worden waarschijnlijk veroorzaakt door deletie van één en hetzelfde gen op chromosoom 15.

X

Van demokratie in het ziekenhuis is pas dan sprake wanneer de directie elke vier jaar op een programma gekozen zou worden.

XI

De geringe animo onder artsen om zich te specialiseren tot neonatoloog is mogelijk te wijten aan de slechte werkomstandigheden.

Aangezien assistenten in opleiding (AIO's) gedurende de vier jaar, waarin zij geacht worden te promoveren, zwaar onderbetaald worden, wordt hen aangeraden hun proefschrift in de vorm van een syllabus uit te geven.